Stress regulating systems and their role for an intrauterine origin of diseases

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Summary

Epidemiological data strongly suggest that an unfavorable intrauterine environment may be highly predictive for the risk to develop diseases such as hypertension and the metabolic syndrome ("Fetal origin hypothesis" or Barker Hypothesis (Barker, Osmond et al. 1989). While this correlation has been demonstrated in different populations, the mechanisms underlying this phenomenon are under ongoing investigation. It is hypothesized that intrauterine environmental factors permanently alter gene expression via imprinting mechanisms during sensitive periods of fetal development leading to permanent alteration of the balance and function of endocrine systems.

Two endocrine systems, predominantly involved in the adjustment of the organism towards increased strain, namely the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) are believed to be sensitive towards imprinting during intrauterine life. These two systems represent putative key candidates to be involved in the intrauterine programming of the metabolic syndrome. Indeed, at the time of their own development, these systems are already challenged if adverse intrauterine conditions are present. Later in adulthood, the same systems are actively involved in the pathogenesis of hypertension and the metabolic syndrome. Therefore, it is speculated and animal models provide strong evidence that these systems may undergo developmental alterations during adverse intrauterine conditions leading to permanent changes in their physiological balance and reactivity towards demand.

Our research has focused on the question whether adverse intrauterine conditions activating the human fetal stress systems have programming effects on these systems. Therefore, we

studied two typical, frequently occurring situations of an interference with the function of the human HPA-axis and SNS:

First, fetal growth restriction, resulting mainly from placental insufficiency, exposes the fetus to stress and consecutively to an activation of the HPA-axis and the SNS and second, the iatrogenic application of synthetic corticosteroids in pregnancies at risk for preterm delivery to promote fetal lung maturation suppresses the fetal HPA-axis. Both stimuli alter the fetal HPA-axis regulation at a sensitive time in development and may entail long-term consequences for the fetus. We hypothesized that an intrauterine malprogramming of the fetal HPA-axis and/or the SNS occurs in placental insufficiency and intrauterine corticosteroid application. To test this hypothesis we conducted our studies in human neonates when the influence of placental insufficiency and short term iatrogenic corticosteroid application were no longer present. We analyzed cortisol and cortisone levels from newborn salivary which is an established method to study HPA axis activity. Furthermore, we analyzed 24h holter ECG tracings for various frequency- and time domain parameters of heart rate variability and electrophysiological findings were supplemented by salivary α-amylase measurements as surrogate for cardiac autonomic regulation.

Our first studies analyzed the effect of intrauterine fetal malnutrition as reflected by small for gestational age (SGA) neonates. For this purpose we measured cortisol levels of newborns at the 4^{th} day of life before and after the heel-prick test which is an established stressor for neonates. Interestingly, we found a distinct suppression of HPA axis reactivity in SGA neonates in response to the stress event. Our finding provide evidence for a dysfunction of the ability for SGA neonates to adequately react to a stressful environment and suggests that the HPA axis may undergo permanent alteration during adverse intrauterine conditions.

In contrast to our findings for the HPA axis, analysis of the cardiac SNS did not exhibit any alteration in the cardiac sympathetic-parasympathetic balance. In fact, all electrophysiological

parameters for time- and frequency domain analysis were comparable to control infants and α-amylase levels were not significantly different. Accordingly, the balance of the cardiac SNS appears to be preserved in the neonatal period of SGA infants thus making an intrauterine re-setting of this system rather unlikely.

Our next step was to analyze whether exogenous glucocorticoids may have the same effect on fetal HPA and SNS axis development as intrauterine growth restriction induced interference with the HPA axis. Glucocorticoids have experimentally been shown to be actively involved in developmental steps and maturation of organ systems. Maternal application of synthetic glucocorticoids is utilized as a therapeutic procedure in pregnancies at risk for preterm delivery to promote fetal lung maturation thus reducing morbidity and mortality of preterm delivered infants significantly. Accordingly, we analyzed the HPA axis of neonates that had received a single course of antenatal betamethasone treatment at 24-34 weeks of gestation due to impending preterm delivery but ultimately continued the pregnancy near or to term. We found that a single course of betamethasone treatment between 24 and 34 weeks of gestation did result in a significant suppression of stress reactivity in healthy neonates more than 6 weeks after treatment. Again, we analyzed parameters of cardiac SNS activity in these infants. Similar to SGA infants, these neonates exhibited a preserved cardiac SNS balance.

From the results of our data it appears that intrauterine HPA axis development is vulnerable towards conditions that alter endogenous glucocorticoid homeostasis and these alterations persist into postnatal life thus suggesting a permanent imprinting of this system. In contrast, stress induced intrauterine activation of the SNS does not directly persist into postnatal life in SGA infants and neonatal sympatho-vagal balance after exogenous glucocorticoid treatment seems to be preserved. Thus, the HPA axis but not the SNS presents a prerequisite to be a regulatory system primarily involved in the intrauterine origin of adult diseases.

Identifying regulatory systems actively involved in the fetal origins hypothesis may provide options for early intervention and disease prevention. The early postnatal period provides a unique time window to identify permanently altered regulatory systems as potential targets because temporary activated mechanisms will normalize in response to a normal environment after birth. Besides, early childhood may still contain some degree of plasticity of these systems with putative options for intervention or re-programming.

Introduction

The intrauterine and neonatal environment determines the vulnerability of the fetus to chronic diseases. This concept has been pioneered by Günter Dörner more than 30 years ago who provided evidence that certain hormone levels play a significant role in the developmental determination of the balance of neuroendocrine systems regulating fundamental functions of the organism. Accordingly, non-physiological hormone levels during vulnerable periods of system development have been suggested to result in the alteration of systems regulating basic needs for survival such as food intake, stress response and reproduction (Dorner 1983). Dörner introduced the term "perinatal programming" to describe the developmental determination of the functional ranges of these key regulating systems. This concept received wide international recognition after the group of David Barker and Nicholas Hales asserted, based on their epidemiological studies, that intrauterine growth was inversely related to cardiovascular and metabolic disease in later life, also known as "Barker hypothesis" (Barker, Osmond et al. 1989; Hales and Barker 1992; Barker 1998). Subsequently the term "fetal programming" was internationally established to describe the notion that certain conditions or events during fetal development cause sustained effects on cellular function and regulatory system balance that ultimately transform into long-term consequences for the organism.

Adaptation of the organism to a certain environment during a period of developmental determination of functional ranges of regulatory systems may provide an evolutionary short-term advantage in terms of reproduction if the perceived postnatal environment comes true but can be detrimental for long-term survival when postnatal conditions do not match the antenatal setting of biological systems. Albeit regulatory systems will maintain some degree of plasticity during life, the prevailing modification of systems may take place during early life.

In the animal, there are various examples for a favorable intrauterine adaptation to environmental conditions to improve survival. As such, the coat of the meadow vole at birth is thicker in the offspring born in autumn than in those born in spring in an experimental setting where in utero and nest temperatures are similar thus providing no intrauterine advantage (Lee and Zucker 1988). As a process of developmental plasticity, postnatal coat thickness has been determined in utero induced by maternally derived signals of day length (Lee, Spears et al. 1989). Likewise, unfavorable intrauterine conditions have the potential to influence regulatory systems in order to provide an immediate survival advantage. As an example, in the condition of placental insufficiency, fetal demand exceeds maternal supply for oxygen and nutrition. To survive, the fetus has to adapt by adjusting energy distribution to organs important for survival and by reducing growth rate. If these events occur during a window of developmental plasticity of regulatory systems, the biological tradeoff, however, may be permanent alterations in the balance of these systems, ultimately not matching the predicted environment after birth. Indeed, intrauterine malnutrition has been related to the risk for hypertension, insulin resistance, type-2 diabetes and cardiovascular disease in later life (Gluckman, Hanson et al. 2008).

Unfavorable intrauterine environment and the risk for metabolic and cardiovascular diseases

The concept of an intrauterine origin of adult diseases derives from epidemiological studies that have linked fetal growth with common adult disorders. As such, Barker et al. (Barker, Winter et al. 1989) were the first to show that body weight at birth, a rough parameter for conditions during fetal life, was inversely correlated with the risk for men to die from cardiovascular disease in later life. They analyzed a cohort of 5654 men born in Hertfordshire, England, between 1911 and 1930. These findings were confirmed by a Swedish study in 15000 men and women with a 97% follow up of at least 50 years that revealed an increase in

death rates from ischemic heart disease in individuals with birth weights within the lower quartiles as compared to the highest quartiles (Leon, Johansson et al. 2000). An American study retrospectively analyzed more than 70000 women as part of the Nurses study and found a strong association between self-reported birth weight and coronary heart disease and stroke (Rich-Edwards, Stampfer et al. 1997). Further studies (Frankel, Elwood et al. 1996; Frankel, Elwood et al. 1996; Martyn, Barker et al. 1996; Stein, Fall et al. 1996) with few exceptions (Vagero and Leon 1994; Hulman, Kushner et al. 1998) confirmed these findings. As such, the association of birth weight and cardiovascular disease could be reproduced in populations of different ethnic origin, age and sex (Osmond, Barker et al. 1993; Frankel, Elwood et al. 1996; Stein, Fall et al. 1996; Leon, Lithell et al. 1998). These findings appear to be independent from prematurity originated abnormal birth-weight (Barker, Osmond et al. 1993).

Associations could also be found for blood pressure as major risk factor for cardiovascular disease. A systematic review of 80 studies including more than 400000 individuals found low birth weight and small head circumference as significant risk factor for an increase in systolic blood pressure (Huxley, Shiell et al. 2000). However, methodical considerations and confounding factors have been under debate (Huxley, Neil et al. 2002; Hardy, Sovio et al. 2006).

Similarly, evidence has been found for an association of an adverse intrauterine environment and impaired glucose tolerance, insulin resistance, type 2 diabetes and hyperlipidemia. (Hales, Barker et al. 1991; Newsome, Shiell et al. 2003; Davies, Smith et al. 2004; Lapidus, Andersson et al. 2008). The group of Barker demonstrated in a collective of 438 men from the Hertfordshire collective born between 1920-1930 that lower birth weight was related to an increase in impaired glucose tolerance and non-insulin dependent diabetes (Hales, Barker et al. 1991). These findings were confirmed in different populations of men and women. As such, the prevalence of impaired glucose tolerance or diabetes fell from 27% in individuals

with a birth weight of 2.5 kg or less to 6% who weighed more than 3.41 kg after adjusting for current body mass index (BMI) (Philipps, Barker et al. 1993). Similar results were found in a Dutch and American study (Ravelli, van der Meulen et al. 1998; Rich-Edwards, Colditz et al. 1999) and confirmed in a large literature review (Newsome, Shiell et al. 2003).
Subsequently, a wide range of diseases including schizophrenia and depression (Thompson, Syddall et al. 2001), osteoporosis (Dennison, Syddall et al. 2005) and obstructive pulmonary disorders (Villamor, Iliadou et al. 2009) have been suggested to be linked with poor fetal growth.

Birth weight as risk factor for adult disease, however, appears to be U-shaped because large size at birth, as evidence for excessive energy supply, equally adds to the risk for metabolic disorders (Rich-Edwards, Colditz et al. 1999; Osmond and Barker 2000; Boney, Verma et al. 2005) and probably to cancer (Okasha, Gunnell et al. 2002; Vatten, Maehle et al. 2002; McCormack, dos Santos Silva et al. 2003; Kajantie, Osmond et al. 2005). Furthermore, it is noteworthy to mention, that at least for the risk of impaired glucose tolerance and the metabolic syndrome, not only intrauterine - but also early postnatal conditions of nutritional supply appear to be highly relevant (Plagemann, Heidrich et al. 1992; Plagemann 2004; Plagemann 2006).

All these associations have been shown to be largely independent from classical life-style risk factors such as smoking, body mass index (BMI) or sedentary life which add to the effect of birth weight.
Thus, in contrast to the classic understanding of the development of “life-style” diseases where multifactorial disorders such as cardiovascular and diabetic diseases are influenced in the context of a complex genetic background by external factors during childhood, adolescence and adulthood (“life-style”), the fetal programming theory proposes a significant,

independent influence of conditions during fetal life on the later health status additional to postnatal environment and genetics (Figure 1).

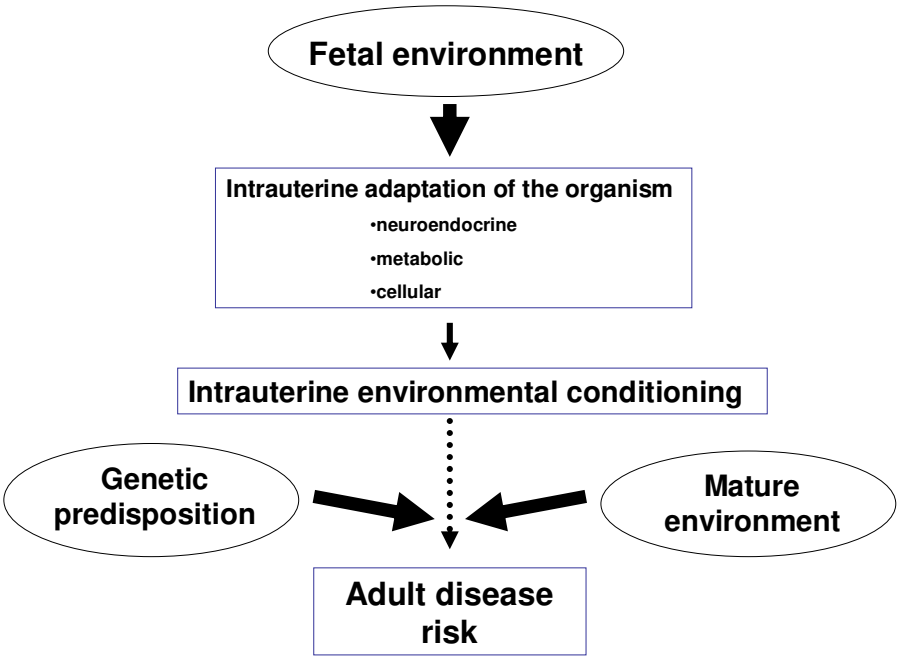

Figure 1: The concept of fetal origins of adult diseases

Adverse intrauterine conditions that induce intrauterine programming

Size at birth is not causal in the pathway to disease. Birth weight and other anthropometric indices are rather unspecific symptoms of interactions between the genomic background and environmental intrauterine conditions during fetal development. An important reason for a fetus not to reach its genetic growth potential is impaired oxygen and nutrient supply. Thus, various experimental animal models have been applied ranging from maternal dietary and protein restriction (Snoeck, Remacle et al. 1990; Blondeau, Garofano et al. 1999; Manning and Vehaskari 2001; Ozaki, Nishina et al. 2001) to reduced uteroplacental blood flow (Simmons, Templeton et al. 2001; Payne, Alexander et al. 2003) leading to impaired fetal

growth with the endpoint of cardiovascular, metabolic and endocrine abnormalities before and after birth.

In the human, intrauterine growth restriction (IUGR) complicates approximately 5% of all pregnancies in developed countries (Vandenbosche and Kirchner 1998). Malfunction of the placenta due to disturbed trophoblasts spreading (Kaufmann, Black et al. 2003) is the leading cause for undernutrition in the developing fetus. The fetus survives by adaptation of metabolic, endocrine and cardiovascular systems by activating a sparing mechanism for optimal homeostasis under these conditions leading to preservation of key body organ functions relevant for survival but at the cost of energy consuming fetal- and organ growth (Bauer, Walter et al. 2003). The result is an infant at an increased risk for intrauterine growth restriction and the deleterious consequences such as intrauterine asphyxia or death.

Excessive intrauterine growth represents the other end of an U-shaped birth weight risk curve for adult hypertension and the metabolic syndrome. Pathologic macrosomia in terms of intrauterine environment derives from excessive overnutrition of the fetus commonly caused by an altered glucose homeostasis of an adipose or diabetic mother leading to fetal hyperinsulinemia and fat deposition resulting in alterations of glucose and lipid metabolism (Catalano, Presley et al. 2009).

Various insults during pregnancy with- or without significantly altering gross birth weight, however, may have the potential to affect the development of biologic homeostasis in the fetus. As such, maternal antenatal stress (Van den Bergh, Mulder et al. 2005), exposure to synthetic glucocorticoids (Seckl and Holmes 2007), altered diet or maternal smoking (Wideroe, Vik et al. 2003) all appear to have the potential to induce hypertension, insulin resistance and other symptoms of the metabolic syndrome.

Biological basis of intrauterine programming

Adaptation of the developing organism to a certain environment can occur on different levels of the biological system involving cellular function profiles, organogenesis, altered tissue differentiation and endocrine mechanisms. There has been accumulating evidence within recent years that epigenetic mechanisms may play a key role in the transformation of these adaptive processes into permanent long term effects with putative transgenerational impact.

Epigenetic mechanisms

Epigenetics describes the process where DNA transcription is permanently modified without altering the sequence of the nucleotids within the DNA molecule. This process is mainly mediated by methylation of CpG dinucleotids of the promoter regions of specific genes and by deacetylation resulting in altered histone packing of the chromatin controlling the access of transcription factors to the DNA sequence (Jaenisch and Bird 2003; Goldberg, Allis et al. 2007) Furthermore, chromatin influencing non-coding RNA is believed to be involved in control of epigenetic mechanisms (Bernstein and Allis 2005).

There is evidence that during cellular differentiation, epigenetic modification of gene expression through environmental conditions is possible and once established, leading to permanent alteration of tissue-specific gene expression that may even be heritable. Epigenetic mechanisms are gene- and cell type specific. As such, an altered p53 CpG gene methylation of the renal p53 promoter has been found in the rat as a result of uteroplacental insufficiency leading to increases in apoptosis and reduced nephron numbers in the full-term rat kidney (Pham, MacLennan et al. 2003). In the human, kidney size and nephron numbers are reduced in individuals born as SGA (Hughson, Farris et al. 2003; Schmidt, Chellakooty et al. 2005). A

low nephron number has been suggested to be involved in the intrauterine programming of hypertension (Schreuder and Nauta 2007).

Bogdarina et al. (Bogdarina, Welham et al. 2007) were able to show in the rat model, that a maternal low protein diet significantly altered the methylation of the Angiotensin (AT 1b) receptor gene promoter resulting in increased receptor protein expression in the adrenal gland of the offspring leading to increased adrenal Angiotensin responsiveness. This effect has also been suggested to be involved in development of hypertension in the offspring.

Furthermore, unbalanced maternal nutrition induces epigenetic alterations in the methylation status and expression of the glucocorticoid receptor and peroxisomal proliferator-activated receptor (PPAR) involved in metabolic processes in the rodent liver (Lillycrop, Phillips et al. 2005). Histone deacetylation appears to contribute to the observed epigenetic effect of type 2 diabetes programming. The pathogenesis of pancreatic β-cell failure in IUGR rats has been related to epigenetic induced permanent suppression of Pdx-1 expression, a gene playing a critical role in normal β-cell function (Simmons 2007). In addition, intrauterine malnutrition results in a significant reduction of pancreatic β-cell mass (Garofano, Czernichow et al. 1998; Reusens and Remacle 2006).

Since epigenetic alteration of DNA expression is generally stable during mitosis, these modifications are maintained during life. Programming effects, however, may not become apparent until later in life, especially if they are involved in responses to later environmental challenges at a biological phase when compensating mechanisms collapse as a result of aging.

Epigenetic mechanisms may not only act as mediators of developmental plasticity but may also induce a transgenerational transmission of intrauterine induced system alterations. As such, in the rat, protein restriction in the F 0 generation was able to induce an altered promoter methylation of hepatic genes in the F 2 generation (Burdge, Slater-Jefferies et al. 2007) and

antenatal glucocorticoid treatment transferred into elevated hepatic PEPCK and insulin levels in the 2nd generation (Drake, Walker et al. 2005).

The role of glucocorticoids

There is major experimental evidence that maternal as well as fetal glucocorticoids play a key role in prenatal programming and recent evidence suggests that these effects might at least in part be transferred through epigenetic mechanisms. The notion of the importance of glucocorticoids is further supported by their potency to promote maturational processes during organogenesis that can be induced by exogenous glucocorticoid administration as well (Bian, Seidler et al. 1992; Bian, Seidler et al. 1993; Cole, Blendy et al. 1995; Fowden 1995). Indeed, major physiologic systems involved in the development of hypertension and the metabolic syndrome are glucocorticoid sensitive targets during intrauterine development (Seckl and Meaney 2004; Drake, Tang et al. 2007).

Programming effects of glucocorticoids can be simulated in the animal by administration of synthetic glucocorticoids with unhindered placental transfer or by blocking the placental 11-beta hydroxysteroid dehydrogenase (HSD) -2. As in the IUGR placenta, biological potent maternal cortisol cannot be adequately converted to its inactive metabolite, cortisone, due to a decreased placental 11-beta HSD-2 capacity which acts as a placental barrier to maternal glucocorticoids (Dy, Guan et al. 2008).

In the rat, maternal antenatal dexamethasone treatment results in elevated blood pressure in the offspring (Benediktsson, Lindsay et al. 1993; Woods and Weeks 2005). Likewise, hypertension during adulthood could be induced by maternal administration of dexamethasone in the sheep (Dodic, May et al. 1998; Dodic, Hantzis et al. 2002).

Interestingly, inhibition of increased maternal glucocorticoid synthesis during nutritional restriction by metyrapone treatment prevented development of hypertension in the offspring whereas glucocorticoid supplementation of these metyrapone treated dams restored the hypertensive effect (Langley-Evans 1997; Langley-Evans 1997). Hypertensive effects could also be induced by blocking the placental 11-beta-HSD-2 using carbenoxolone. These effects are proven to be glucocorticoid dependent as adrenalectomized dams would deliver normotensive offspring (Lindsay, Lindsay et al. 1996). The role of glucocorticoids could further be enforced by the finding that adrenalectomy in intrauterine "programmed" rats restored normal blood pressure levels (Gardner, Jackson et al. 1997).

These glucocorticiod effects have been shown to be transferred in a number of organ systems including the renal system and the renin-angiotensin-aldosterone system (RAAS) (Ortiz, Quan et al. 2003; Wintour, Moritz et al. 2003; Dickinson, Walker et al. 2007; Shaltout, Figueroa et al. 2008) as well as the cardiovascular system (Molnar, Howe et al. 2003; Roghair, Segar et al. 2005; Hadoke, Lindsay et al. 2006; Atanasova, Wieland et al. 2008; Roghair, Miller et al. 2008) and at the level of central nervous centers (Dodic, Peers et al. 1999).

Glucocorticoids are also involved in programming of glucose tolerance and metabolism. Again in the rat as well as in the sheep model, persistent alterations in glucose and insulin levels have been found in adult offspring after increased endogenous or exogenous intrauterine glucocorticoid exposure (Lindsay, Lindsay et al. 1996; Nyirenda, Lindsay et al. 1998; Moss, Sloboda et al. 2001; De Blasio, Dodic et al. 2007). Effects of carbenoxolone could similarly be prevented by maternal adrenalectomy confirming glucocorticoid dependency of these effects (Lindsay, Lindsay et al. 1996). Likewise, fat metabolism and adipositas can be glucocorticoid induced (Bispham, Gopalakrishnan et al. 2003; Cleasby, Kelly et al. 2003; Gnanalingham, Mostyn et al. 2005). Mechanisms include impaired pancreatic β-cell function (Shen, Seckl et al. 2003) and pancreatic development (Gesina,

Blondeau et al. 2006), altered expression of hepatic metabolic enzymes (Nyirenda, Lindsay et al. 1998) and glucose and fatty acid metabolism in the muscle and adipocyte (Budge, Gnanalingham et al. 2005; Wyrwoll, Mark et al. 2008).

Modulation of glucocorticoid action is mediated through glucocorticoid receptors. Of note, the promoter region of the glucocorticoid receptor contains a transcriptionally active CpG region sensitive to methylation / demethylation. At the same time, glucocorticoids have a demethylating capacity (Zhavoronkova and Vaniushin 1987). Accordingly, glucocorticoid effects are suggested to induce epigenetic effects as well as are dependent on epigenetic control of the glucocorticoid receptor expression which occurs in a tissue specific manner (McCormick, Lyons et al. 2000; Thomassin, Flavin et al. 2001; Weaver, Cervoni et al. 2004).

Involvement of neuroendocrine and neurohumoral systems

Induction of biological long-term effects are most likely conferred by homeostasis regulating systems that are activated during adverse intrauterine conditions.

Periods of unfavorable intrauterine conditions such as placental insufficiency lead to an activation of the organism´s stress systems, namely the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). As a consequence, the developing fetus is exposed to increased levels of glucocorticoids and catecholamines mediating protective reactions of the organism. Thus, the HPA axis and the SNS are strong candidates to be involved in the programming as well as being programmed by these conditions.

The HPA axis: Target and mediator of intrauterine programming

The HPA axis represents the organism´s central organ of stress regulation. Activation of the HPA axis leads to an increase in adrenal cortisol release thereby communicating environmental challenges to the fetus to optimize the organism for survival. Serum levels of cortisol have been shown to be significantly increased in human fetuses with IUGR (Economides, Nicolaides et al. 1988; Goland, Jozak et al. 1993). Increased levels of cortisol represent an important developmental challenge during organogenesis with tissue specific consequences as has been shown above.

Increased cortisol levels of fetal as well as of maternal origin due to increased placental transfer in IUGR but also fetal exposure to synthetic glucocorticoids appear to have a considerable impact on regulation of the developing fetal HPA axis itself.

As such, the HPA axis and its key limbic regulator, the hippocampus are especially sensitive to glucocorticoids as part of a tightly regulated feedback mechanism involving glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at the level of the hippocampus, hypothalamus and pituitary gland to inhibit HPA activity (Jacobson and Sapolsky 1991).

Experiments in the rat have shown that intrauterine overexposure to glucocorticoids results in attenuated hippocampal GR and MR expression. The resulting permanently increased cortisol levels and hypertension in adulthood suggests an impaired negative feedback sensitivity of the HPA axis (Levitt, Lindsay et al. 1996). On the other hand, the amygdale, a center for anxiety and fear modulation that is interconnected with HPA axis dependent stress regulation via corticotrophin releasing hormone (CRH) signaling (Feldman and Weidenfeld 1998) is vulnerable to antenatal glucocorticoid excess as levels for CRH are permanently increased in the adult rat as well as GRs and MRs, pointing to an increased excitatory activity on the HPA axis (Welberg, Seckl et al. 2000; Welberg, Seckl et al. 2001). Likewise, the hypothalamus has been shown to express increased levels of CRH mRNA and protein after intrauterine undernutrition in the rat (Nunez, Ruiz et al. 2008) and GRs but not MRs have been found to

be permanently decreased in the hypothalamus and the pituitary gland (Bertram, Trowern et al. 2001; Hawkins, Hanson et al. 2001) (Figure 2).

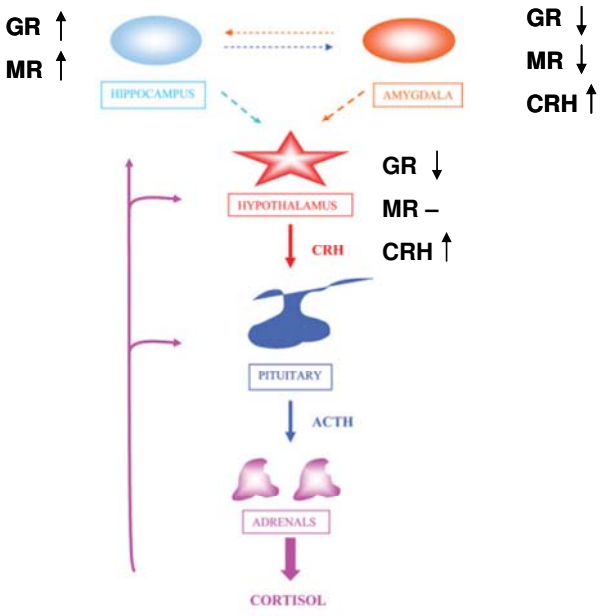

Figure 2 A possible schema of glucocorticoid (GR) and mineralocorticoid (MR) receptor programming of the HPA axis. Adapted from (Drake, Tang et al. 2007)

Accordingly, permanent alterations of these receptors may induce a resetting of the balance and reactivity of the HPA axis in later life. Nevertheless, HPA axis regulation and programming is complex and far from being completely understood as factors such as gender (McCormick, Smythe et al. 1995), species specific differences (Matthews 2002) and different time windows for vulnerability of HPA axis development (Fowden, Giussani et al. 2005) further add to the complexity of the system. Of note, the GR appears to be susceptible to epigenetic alteration (Weaver 2007).

In conclusion, the HPA axis is strongly involved in regulation of cardiocirculatory and metabolic homeostasis of the organism. Permanent alteration of its set-point and balance throughout life will ultimately impact adult health. As a matter of fact, elevated cortisol levels have been linked to arteriosclerosis and diabetes (Sapolsky, Romero et al. 2000).

The mutual dependence of the HPA axis and the sympathetic nervous system

The HPA axis is closely interconnected with the sympathetic nervous system (SNS) and both are subject to environmental input during intrauterine life. As such, catecholaminergic neurons ascending from the medulla exert stimulatory effects on CRH neurons located in the hypothalamic paraventricular nucleus (PVN) via α1 adrenergic receptors (Herman, Prewitt et al. 1996). Furthermore, hippocampal corticoid receptor levels modulating glucocorticoid negative feedback are modified by catecholaminergic brainstem neurons (Barbazanges, Piazza et al. 1996). On the other hand, glucocorticoids provide direct signals for noradrenergic system maturation in the brainstem and thus on central noradrenergic activity (Slotkin, Lappi et al. 1992). Likewise, cardiac noradrenergic innervation is affected by glucocorticoids (Bian, Seidler et al. 1993).

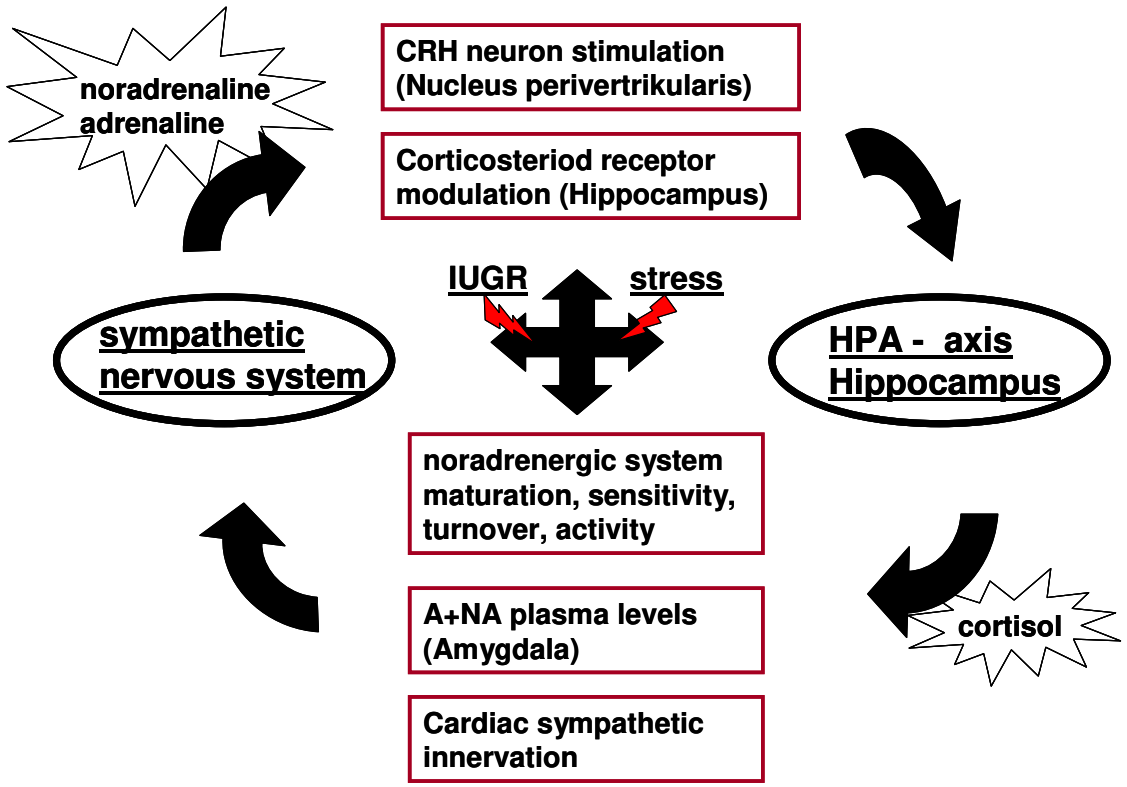

Figure 3 Interaction between the HPA –axis and the sympathetic nervous system

The sympathetic nervous system

The SNS is, as the HPA axis, activated during periods of intrauterine strain (Economides, Nicolaides et al. 1991; Westgren, Lingman et al. 1997). On the other hand, adrenergic cardiovascular drive and noradrenergic influences play a key role in the development or progression of hypertension and the metabolic syndrome in the adult (Mancia, Bousquet et al. 2007; Grassi, Arenare et al. 2009)

The SNS is composed of multiple function-specific subunits (Janig and McLachlan 1992) and programming of sympathetic nervous system function is believed to occur regionally rather than on a global basis, suggesting that subdivisions of the sympathetic nervous system may be influenced by different sets of environmental variables (Young 2002). It has been suggested, that exposure dependent activation of specific sympathetic pathways during development may prevent these subdivisions to undergo nerve cell loss thereby determining the structure and function of the system (Young 2006).

An increased resting pulse rate in adults with low birth weight has been suggested to be an imperfect measure of an activated SNS as a result of intrauterine system alteration (Phillips and Barker 1997; Flanagan, Vaile et al. 1999). Interestingly, pulse rate was inversely associated with insulin sensitivity in men pointing to the interconnection between sympathetic system activity and glucose metabolism (Flanagan, Vaile et al. 1999). Furthermore, markers of impaired fetal growth were related to autonomic cardiovascular control in adults involving modulation of both sympathetic and parasympathetic function (Ijzerman, Stehouwer et al. 2003; Ward, Moore et al. 2004; Jones, Beda et al. 2007).

Animal models with different experimental settings of intrauterine demand have been described to impact sympathoadrenergic system development that ultimately results in a characteristic phenotype during adulthood. As such, different forms of intrauterine stress,

such as hypoxia, environmental temperature, maternal glucose homeostasis and nutrition have the potential to permanently impact sympathetic system regulation or sympathetic innervation either directly or through indirect pathways such as glucocorticoid induced (Young 2006).

It has been speculated, that an unfavorable intrauterine environment may be a stimulus to promote sympathetic hyperinnervation of fetal vessels and tissues or may enhance catecholamine turnover in developing sympathetic neurons (McMillen and Robinson 2005). Indeed, increased levels of noradrenaline were found in hypothalamic tissue of growth restricted female adult rats (Jansson and Lambert 1999) and an enhanced sympathetic innervation of arteries was observed in the chronically hypoxic chick embryo (Ruijtenbeek, le Noble et al. 2000). Furthermore, prenatal hypoxia lowered adult autonomic nervous activity in cardiac related structures such as the stellate ganglion, the heart and the adrenals. Albeit heart rate and mean arterial pressure during resting conditions did not significantly differ from control animals, during a stress test, these animals revealed a significant increase in heart rate and its variability (Peyronnet, Dalmaz et al. 2002). Others have found a significantly increased basal cardiac sympathetic neuronal activity in newborn growth restricted rats (Shaul, Cha et al. 1989) whereas persistent abnormalities of cardiac noradrenergic innervation was observed after exogenous glucocorticoid exposure (Bian, Seidler et al. 1993).

In summary, the SNS appears to be susceptible to intrauterine influences that induce permanent alterations of its balance. This mechanism may therefore be relevant for the pathogenesis or progression of adult cardiovascular and metabolic diseases.

Conclusions and general aims of our studies

The wealth of studies both in humans and animals that clearly indicate, that the intrauterine environment evolves as a significant determinant for future health or disease has led to a new understanding of the development of diseases of cardiovascular and metabolic origin that have previously been attributed in a large part to "life-style" influences. The newly established paradigm now placed the period of pregnancy at a prominent position of consideration with respect to public health issues and disease prevention and may have a large impact on socioeconomic issues.

While epidemiologic evidence of this interconnection is overwhelming, the exact mechanisms involved in intrauterine imprinting are under extensive investigation.

Two major regulatory units involved in stress regulation, namely the HPA-axis and the SNS, amid others, are studied extensively in various experimental settings of animal models. If indeed these systems would be permanently altered depending on their intrauterine environment, they may represent key elements for the individual risk profile of health. While models may confirm this hypothesis in the animal setting, there is considerably less data in humans. As a matter of fact, adults born small for gestational age (SGA) have been shown to have higher resting cortisol levels and an increased heart rate compared to control populations as evidence for HPA axis- and SNS activation (Flanagan, Vaile et al. 1999; Phillips, Walker et al. 2000). At the same time, these conditions are risk factors in the pathogenesis of cardiovascular and metabolic events. However, this does not automatically imply that the alteration of these systems is of intrauterine origin according to the hypothesis of fetal programming. Since the progression towards disease will involve action of primary- and reaction of compensatory systems, initial factors for disease development have to be identified if preventive measures or therapeutic strategies are to be developed.

According to these considerations, we conducted our studies in the human neonate for two major reasons: 1. To provide data on the human situation as animal models are hampered by the fact that developmental frames cannot always be easily transferred and 2. to analyze a period when temporary up-regulated systems normalize in response to a normal postnatal environment, whereas permanently altered systems may become apparent. Furthermore, this time point is interesting since compensatory system reactions may not have had the time to develop.

If indeed the HPA axis and the SNS experience permanent alterations of intrauterine origin, they should be detectible already shortly after birth.

Considering the fact that these alterations may be mild and clinically not apparent during early life due to a high compensatory reserve being only "exhausted" over many years, we aimed to not only analyze HPA and SNS balance under resting conditions but also to challenge these systems.

Accordingly the following questions were formulated:

1. Do neonates that are SGA exhibit alterations of HPA axis balance during resting or activated conditions that may be attributable to an intrauterine origin (Schaffer, Muller-Vizentini et al. 2009)?

2. Are there signs for the cardiac subunit of the SNS to be shifted in the balance between sympathetic and parasympathetic activity in SGA neonates and may they be exposed during strain (Schaffer, Burkhardt et al. 2008)?

3. If glucocorticoids play a significant role in the development and maturation of the HPA axis and the SNS, do exogenous synthetic glucocorticoids that are administered

as a standard procedure in pregnancies at risk for preterm delivery have the potential to disturb normal HPA axis and SNS development in a way that predisposes these infants to the risk of adult disease development (Schaffer, Luzi et al. 2009) + Schaffer, Burkhardt et al, submitted)

Presented publications

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Blunted Stress Response in Small for Gestational Age Neonates

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ABSTRACT: There is evidence that adverse conditions during intrauterine development affect future health of the offspring. Hypothalamus-pituitary-adrenal (HPA) axis dysregulation is assumed to play an important role in the association of small for gestational age (SGA) and the pathogenesis of hypertension and the metabolic syndrome. Stress response patterns in SGA neonates may identify a link with intrauterine-induced permanent maladaptation of the HPA axis. Salivary cortisol and cortisone levels were therefore analyzed during resting conditions and in response to a pain-induced stress event in SGA (<5th percentile) and appropriate for gestational age (AGA) neonates born ≥34 wk of gestation. In AGA neonates, salivary cortisol and cortisone levels significantly increased after the stress event ($p < 0.05$). In contrast, SGA infants exhibited a blunted steroid release after stress induction ($p = 0.76$, $p = 0.65$, respectively). No influence of mode of delivery ($p = 0.93$), gender ($p = 0.21$), and gestational age ($p = 0.57$) on stress response patterns was observed in a multiple stepwise regression. SGA neonates show a blunted physiologic activation of the HPA axis in response to a stress stimulus. Thus, intrauterine-induced alteration of HPA axis regulation seems to persist into the postnatal period and represents a prerequisite for the hypothesis of HPA axis involvement in the fetal origin of adult diseases. **(*Pediatr Res* 65: 231–235, 2009)**

Substantial evidence from epidemiologic data and experimental animal models indicate that stressful conditions during intrauterine fetal life affect behavioral development and future health of the offspring. Maladaptation of the fetal autonomic regulation has been suggested as a putative predisposing factor in the pathogenesis of cardiovascular, metabolic, and psychiatric diseases in low birth weight (LBW) infants (1–3). This has been reinforced by the discovery of an early programming of the stress response–mediating neuroendocrine system, namely the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (4,5). As such, LBW, a surrogate for fetal malnutrition has been linked with increased adrenocortical hormone activity and activation of the sympathetic nervous system (6–9) in childhood and adult life in independent populations.

A pathophysiologic explanation of this phenomenon described by Barker's group as "fetal programming" (10) has been derived from animal models revealing long-term changes in the molecular expression of steroid receptors within the limbic system (11).

Increases in glucocorticoids, such as cortisol and cortisone, are generally considered as adaptive mechanisms to mobilize energy for survival in response to a stressful event or to danger (12). Prolonged intrauterine exposure to stress, however, may lead to an alteration of the set point of the stress regulatory system resulting in increased levels of cortisol that can negatively effect the functioning of several biologic systems (13,14) and ultimately result in arterial hypertension and diabetes mellitus type II, one of the main risk factors for arteriosclerosis-associated diseases.

Because patterns of stress response in early postnatal life may help to identify prenatally induced alterations in the HPA axis and simultaneously exclude an only temporarily activated HPA axis due to adverse intrauterine conditions, we analyzed the stress reactivity of the HPA axis in small for gestational age (SGA) newborns. Our results provide evidence that the HPA axis is already altered in SGA newborns and may, if these alterations persist permanently, play a role in the intrauterine origin of adult diseases.

MATERIALS AND METHODS

Subjects. The study was approved by the Research Ethics Committee of the University of Zurich and the Federal Ethics Commission of the Canton of Zurich. Written maternal consent was obtained from all participants in the study.

Saliva samples from 40 appropriate for gestational age (AGA) and 18 SGA infants were analyzed. Approximately, 40 SGA infants after 34 wk of gestation were born during the study period (April–September, 2005) in our hospital with 2300 deliveries per year. Healthy controls were recruited until approximately the double number of study patients was reached to obtain adequate numbers for statistical evaluation. SGA was defined as birth weight below the 5th percentile of the gender-specific newborn reference chart (15). Birth weight between the 10th and 90th percentile was required for AGA children. Only healthy newborns delivered after 34 wk of gestation (≥238 d) were included in the study. Newborns requiring intensive care or those with malformations were excluded from the study. Additional exclusion criteria included maternal substance abuse (nicotine, alcohol) and exogenous corticosteroid treatment (*e.g.*, for lung maturation induction) during pregnancy. Sonographic measurements of fetal crown-rump length and biparietal diameter during the first trimester served as parameters for accurate determination of gestational age. In the SGA group, 10 infants had a diagnosis of intrauterine growth restriction (IUGR) established either by serial sonographic biometry or by pathologic Doppler measurements, one was SGA alone and 7 infants were referred to our clinic for delivery only and did not receive antenatal care at our institution. Sonographic data for the latter group were not available.

Experimental procedures. Salivary cortisol and cortisone levels were collected between 72 and 96 h postnatal during a routine heel prick for the Guthrie test (a newborn screening test for metabolic disorders). This was considered to be the equivalent of a stress event. Although infants are born

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Abbreviations: AGA, appropriate for gestational age; **HPA axis,** hypothalamus-pituitary-adrenal axis; **SGA,** small for gestational age

without a circardian rhythm and acquire it only within the first year of life (16), saliva samples were collected only during the morning hours between 8 a.m. and 1 p.m. from each infant 10 min before and 20 min after the stress event. Baseline levels were obtained in an undisturbed environment. The time of collection was based on experiments revealing peak cortisol responses between 20 and 30 min postmanipulation (17). To obtain the saliva, a cotton swab was placed in the infants' mouth for a duration of 5 min. Salivary cortisol reflects the unbound, active fraction of cortisol and is highly correlated with plasma cortisol levels (18,19). Samples were placed in saliva collection tubes (Salivette, Sarstedt, Nümbrecht, Germany) and frozen at −20°C until further analysis.

Cortisol/cortisone measurements. Saliva cortisol and cortisone were determined simultaneously using liquid chromatography tandem mass spectrometry, with atmospheric pressure chemical ionization in the positive ion mode, according to a modified method of Rauh *et al.* (20). One hundred microliters of the samples and calibrators were deproteinized with methanol/zinc sulfate (50 g/L, 1/1 vol/vol). After centrifugation, the supernatants were applied to an online solid-phase extraction column with subsequent high-performance liquid chromatography separation using column switching (extraction column: Oasis HLB 2.1 × 20 mm, 15 μm; Waters, Milford, MA). The samples were washed with 5% methanol and eluted in back-flush with 2 mM ammonium acetate/methanol (30:70, vol/vol) onto the analytical column (Chromolith RP 18e100 × 4.6 mm, Merck, Darmstadt, Germany) at a flow rate of 1 mL/min. Sample analysis was performed in the multiple-reaction monitoring mode with a dwell time of 150 ms per channel using the following transitions for quantification (qualifier transition): *m/z* 363.2/121.2 (363.2/309.4) cortisol, *m/z* 361.1/162.9 (361.1/239.0) cortisone, *m/z* 367.3/121.2 Cortisol-d4.

Statistical analysis. We applied STATA 9 Statistics/Data Analysis Software (Stata Corporation, College Station, TX) for statistical analysis according to Altman's recommendations for repeated measurements (21). Baseline characteristics of SGA and AGA infants were compared using the Mann–Whitney test and χ^2 test when appropriate. Because basal levels of cortisol vary considerably in individual newborns and children (17), absolute values and relative alterations were analyzed. Cortisol and cortisone values were not normally distributed as analyzed by the Shapiro-Francia W' test. We therefore analyzed the difference between cortisol and cortisone baseline levels and time point "20 min post" using the Wilcoxon signed rank test. The Mann–Whitney test was used for comparison of raw baseline cortisol and cortisone levels. A stepwise multiple regression was applied to test for putative influencing factors on cortisol alterations such as gestational age, birth weight independent of gestational age, mode of delivery (vaginal/cesarean section), and gender. To validate our statistical analyses, a power calculation was conducted in SGA infants assuming the mean increase in cortisol levels of AGA infants as relevant. Finally, cortisol and cortisone levels in AGA and SGA infants were tested for correlation using the Spearman rank correlation test. The level of statistical significance of all analyses was set at $p < 0.05$.

RESULTS

The gestational ages of the AGA and SGA newborns were comparable (median: 273 *versus* 266 d of gestation, respectively, $p = 0.29$). Median birth weight of the SGA children was 2265 g corresponding to the 1.6th weight percentile compared with 3288 g corresponding to the 52nd percentile in the AGA group. A summary of the characteristics of the study population is given in Table 1.

Median levels for basal cortisol and cortisone were comparable in the AGA and SGA neonates (1.18 *versus* 0.95 ng/mL, $p = 0.7$; 11.35 *versus* 13.55 ng/mL, $p = 0.42$, respectively). Individual stress responses are illustrated in Figure 1*a* and *b*. In AGA neonates, salivary levels of cortisol and cortisone significantly increased after the stress stimulus (2.4 and 18.15 ng/mL, $p < 0.05$, respectively). In contrast, the cortisol and cortisone response in SGA neonates was noticeably blunted (0.69 ng/mL, $p = 0.76$ and 13.05 ng/mL, $p = 0.65$, respectively) (Fig. 2*a* and *b*). To validate the absent cortisol response in SGA neonates a one-sample t test power calculation was conducted for the given sample size ($n = 18$) revealing a power of 99% at a significance level of 0.05.

Table 1. *Baseline characteristics*

	AGA	SGA	*p*
Gestational age (d)	273 (240–294)	266 (240–292)	0.289
Birth weight (g)	3288 (2100–3780)	2265 (1470–2870)	<0.05
Weight percentile	52 (17.6–91.6)	1.6 (0.1–4.3)	<0.05
Body length (cm)	49.5 (45–54)	45 (40–48)	<0.05
Length percentile	65.2 (5.3–100)	3.85 (0–35.2)	<0.05
Gender, male/female	18/22	6/12	0.697
Delivery, vaginal/cesarean	25/15	6/12	<0.05
5-min APGAR score	9 (8–10)	9 (8–10)	0.307
Maternal age (yr)	30 (18–39)	29 (19–39)	0.839
Parity	2 (1–4)	1 (1–4)	<0.05

Values are medians with ranges in parentheses; $n = 40$ appropriate for gestational age (AGA) neonates and 18 small for gestational age (SGA) neonates.

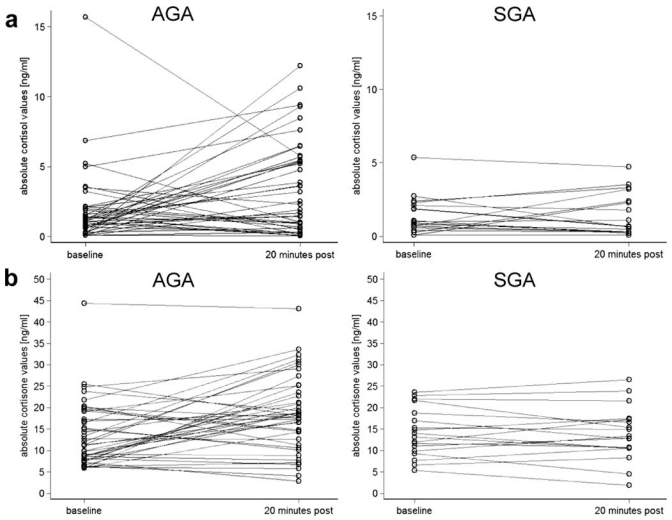

Figure 1. Salivary absolute levels for cortisol (*a*) and cortisone (*b*) including individual courses of AGA ($n = 40$) and SGA ($n = 18$) newborns before (baseline) and after (20 min post) application of the stress stimulus.

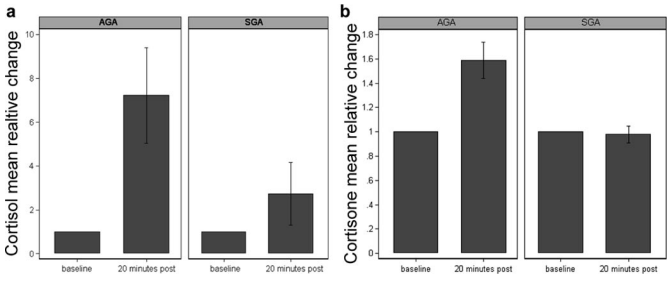

Figure 2. Mean relative salivary level alterations for cortisol (*a*) and cortisone (*b*) and SEM. In AGA newborns ($n = 40$), cortisol and cortisone levels significantly increase after stress induction ($p < 0.05$). In SGA newborns ($n = 18$), cortisol and cortisone levels do not significantly increase after application of the stress stimulus ($p = 0.76$, $p = 0.65$, respectively).

To test for a putative influence of gestational age, gender, birth weight, and mode of delivery on cortisol stress response patterns a multiple stepwise regression model was applied. None of these factors were found to have a significant influence ($p = 0.574$, $p = 0.207$, $p = 0.347$, $p = 0.927$, respectively, Table 2).

Table 2. *Multiple stepwise regression model*

	Regression coefficient	Standard error
Gestational age	−0.0246	0.0458
Birth weight	0.0011	0.0011
Gender	0.8982	0.9786
Mode of delivery	0.0947	1.0211

Stepwise regression of putative confounding factors on HPA axis activity. The stepwise backward regression started with this full model, the criterion for removing a variable was $p \geq 0.200$. Adjusted R^2 for full model: −0.0220.

Finally, a Spearman rank correlation revealed a strong correlation for cortisol and cortisone levels in AGA ($R = 0.77$, $p < 0.001$) and in SGA ($R = 0.82$, $p < 0.001$) neonates, thus excluding the possibility that decreased cortisol responses in SGA neonates could be the result of an increased conversion of cortisol to cortisone (22).

DISCUSSION

We have shown that the hypothalamic-pituitary-adrenal (HPA) axis system of SGA neonates does not adequately react in response to the application of a stress stimulus. Experiments in fetal sheep showed that the cortisol responses to acute stresses are often blunted during suboptimal intrauterine conditions in late gestation (23). In the presence of adverse intrauterine conditions, decreases in the mRNA content of hypothalamic corticotrophin-releasing hormone, pituitary glucocorticoid receptor, and adrenal ACTH (ACTH) receptor have been found (11), which will impair feedback regulation of the axis. These observations suggest that adverse intrauterine conditions alter both the set point and sensitivity of the fetal HPA axis. Alterations of the HPA axis activity in human adults born SGA have been reported repeatedly. Thus, birth weight has been shown to be inversely correlated with basal cortisol levels in independent populations (9). Furthermore, cortisol responses to pharmacological ACTH challenge (24,25) and after psychosocial stress (26) were exaggerated in adults born with LBW. Others, however, did not find a clear inverse correlation between birth weight and HPA activity (27). These inconsistent results may reflect large heterogeneities in the populations studied, often a mixture of premature infants, infants with uncertain gestational ages, LBW fetuses, fetuses with IUGR as well as different experimental settings.

Although the majority of studies in humans provide strong evidence for an alteration of the HPA axis set point and feedback in adults born SGA, these findings do not necessarily represent etiologic factors involved in the concept of fetal programming especially considering the interdependence of the pituitary-adrenocortical response with other neuroendocrine regulatory systems. Moreover, the influence of environmental factors on alterations of the HPA system remains unknown, because this factor was not controlled in most of the above-mentioned studies. To elucidate a putative intrauterine induction of permanent alteration of the HPA-axis system balance, we focused on HPA-axis activity and development in the early postnatal period. In our study, we were able to demonstrate that the stress response is altered in SGA neonates on the 4th day of life. It is difficult to exactly define an adverse intrauterine environment for the fetus. Fetuses with IUGR are not necessarily SGA and the definition of IUGR varies considerably in the literature. The definition of SGA is unequivocal and is commonly referred to as a weight below the 10th percentile for gestational age. If the definition threshold is set low and fetuses with aneuploidies and infections are excluded, a relatively homogenous population of fetuses experiencing adverse intrauterine conditions is left. In our study, we adopted a threshold for SGA definition as birth weight below the 5th percentile. The median birth weight of the SGA newborns in our study corresponds to the 1.6th percentile defining a relatively homogeneous cohort of growth-restricted neonates. The majority of epidemiologic studies have correlated just LBW with the risk for hypertension (28). One may expect an even more pronounced effect if only infants with proven intrauterine compromise could have been included in these studies.

A strength of our study population is that, in contrast to most studies, maternal influencing factors, such as nicotine and alcohol consumption during pregnancy, and antenatal corticosteroid administration that have been shown to induce growth restriction, but may at the same time have independent effects on the HPA axis of the mother and child (29–34), have all been excluded in our study.

The heel-prick test has been shown to be a significant stress event for the newborn, resulting in HPA axis activation as indicated by significantly increasing cortisol levels in response to the test (35). We found a wide variation in cortisol levels between individual infants. This variation has been observed in other studies as well. Nonetheless, variations over time in the same individual have not been shown to vary significantly (17,36).

Few studies analyzed the activity of the human HPA axis in the adolescent and perinatal period. In SGA children, increased plasma and urinary glucocorticoid levels were found at the age of 9 yr (37), others, however, did not observe a correlation between basal serum cortisol levels and rhythms during a 24-h period in 8–10-y-old children born SGA (38).

To our knowledge, the present study is the first to analyze HPA reactivity in healthy human SGA neonates. Experiments in neonates provide the possibility to study the persistence of intrauterine-induced alteration of the HPA axis into the postnatal period and to differentiate them from changes, which are only transiently altered as a compensatory mechanism during intrauterine undernutrition. At the same time, long-term effects resulting from these primary alterations do not yet have the time to develop. Furthermore, the neonatal period may represent an intermediate stage of HPA axis development still susceptible to plastic changes.

The absent stress response in SGA neonates in our study was rather surprising. A delay in the maturation of the HPA system as a possible explanation is rather unlikely, because premature AGA newborns at 34 wk of gestation already display normal stress reactivity (31). Furthermore, maturation of cerebral structures in SGA fetuses is considered to be rather accelerated (39).

Interestingly, children seem to show reduced cortisol responses to stress factors during the first year of life (40,41),

which seems to be a stress hyposensitive period. The absent response to stress induction in our SGA population may therefore reflect an accelerated HPA system maturation in comparison to AGA infants accounting for inadequate time for proper establishment of important developmental steps.

Growth-restricted preterm infants (<32 wk of gestation) seem to have a lower steroidogenic capacity than infants with uninhibited fetal growth in response to ACTH stimulation leading to an insufficient response to stress (42). This may be related to enzyme deficiencies in the adrenal cortex (43). However, ACTH stimulation cannot differentiate adrenal and pituitary causes of a diminished response.

Stress hyposensitive periods at different stages of postnatal development have been observed in animals as well (44). Blunted cortisol responses to ACTH and acute stressors have been observed in fetal sheep during chronic unfavorable intrauterine conditions (23,45). These observations are consistent with changes on the molecular level reflected by alterations in various hypothalamic receptor expression levels (11), resulting in reduced HPA sensitivity. Considering these data, intrauterine fetal stress because of undernutrition may lead to a hyporeactivity of the HPA system to stressors in early life, but seems to be modified by postnatal factors. If this notion could be confirmed, HPA system plasticity during early life would provide the chance to therapeutically influence the system. Indeed, influencing the HPA system in the postnatal period has been shown to be possible at least in animals (46).

It was suggested that mode of delivery may influence infant HPA axis response in response to different stress levels for up to 2 months of age (47) even though in a larger population this finding was less clear (48). Saliva samples in our study were obtained during the 4th day of life after stabilization of birth-related fluctuations (49). According to a multiple stepwise regression model, mode of delivery does not seem to have an influence on the HPA axis response at least in our collective. Furthermore, there is evidence from animal models that females might be more sensitive to HPA axis programming than males (50,51). However, we did not find a gender difference in HPA axis reactivity in our study. Associations between LBW and increased HPA axis activation have also not been found to be different in adult men and women (52), thereby confirming our results. It seems that gender-specific cortisol reactivity in general depends on different levels of corticosteroid-binding globulin and sex steroids (53). We therefore cannot exclude gender-specific hormonal influences that may manifest during adolescence and adulthood in SGA infants.

Our data only provide a glimpse of HPA axis alteration in the SGA neonate. We do not know whether these alterations persist or how they change during life. Nevertheless, they represent a prerequisite if intrauterine conditions *per se* are to play a role in the etiology of adult diseases. Prospective long-term investigations over years are required to prove this hypothesis.

In conclusion, stress response in SGA infants seems to be abnormal beginning as early as the neonatal period. However, the characteristics of these abnormalities are changing constantly during the course of life. Apparently, a hyporeactive HPA axis system in early life is converted to a hyper-reactive system, both being unfavorable. The exact mechanisms responsible for these alterations are still elusive. The apparent plasticity of the system provides hope that therapeutic reprogramming of this system may be a realistic long-term objective.

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Cardiac autonomic balance in small for gestational age neonates

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Cardiac autonomic balance in small-for-gestational-age neonates

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Departments of [1]Obstetrics, [2]Neonatology, and [3]Pediatric Cardiology, University of Zürich, Zürich, Switzerland; and [4]Department of Pediatrics, University of Erlangen, Erlangen, Germany

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Schäffer L, Burkhardt T, Müller-Vizentini D, Rauh M, Tomaske M, Arlettaz Mieth R, Bauersfeld U, Beinder E. Cardiac autonomic balance in small-for-gestational-age neonates. *Am J Physiol-Heart Circ Physiol* 294: H884–H890, 2008. First published December 7, 2007; doi:10.1152/ajpheart.00318.2007.—The cardiac sympathetic nervous system is one putative key factor involved in the intrauterine programming of adult cardiovascular disease. We therefore analyzed cardiac autonomic system activity in small for gestational age (SGA) neonates. Heart rate variability (HRV) from 24-h ECG recordings were analyzed for time-domain and frequency-domain parameters in 27 SGA neonates [median 261 (240–283) days of gestation] compared with 27 appropriate for gestational age (AGA) neonates [median 270 (239–293) days of gestation]. In addition, salivary α-amylase levels were analyzed during resting conditions and in response to a pain-induced stress event in 18 SGA [median 266 (240–292) days of gestation] and 34 AGA [median 271 (240–294) days of gestation] neonates. Overall HRV was not significantly different in SGA neonates compared with AGA neonates (SD of all valid NN intervals: $P = 0.14$; triangular index: $P = 0.29$), and the sympathovagal balance [low frequency (LF)/high frequency (HF)] was similar ($P = 0.62$). Parameters mostly influenced by sympathetic activity did not reveal significant differences: (SD of the average of valid NN intervals: $P = 0.27$; average of the hourly means of SDs of all NN intervals: $P = 0.66$, LF: $P = 0.83$) as well as vagal tone-influenced parameters were unaltered (average of the hourly square root of the mean of the sum of the squares of differences between adjacent NN intervals: $P = 0.59$; proportion of pairs of adjacent NN intervals differing by >50 ms: $P = 0.93$; HF: $P = 0.82$). Median resting levels for α-amylase were not significantly different in SGA neonates ($P = 0.13$), and a neonatal stress stimulus revealed similar stress response patterns ($P = 0.29$). HRV and salivary α-amylase levels as indicators of cardiac autonomic activity were not altered in SGA neonates compared with AGA neonates. Thus, it appears that the intrauterine activation of the sympathetic system in SGA fetuses does not directly persist into postnatal life, and neonatal sympathovagal balance appears to be preserved.

intrauterine programming; cardiovascular; amylase; heart rate variability; sympathovagal balance

THERE IS EVIDENCE that the development of adult cardiovascular disease is initiated by unfavorable conditions during intrauterine fetal life among genetic and environmental factor interactions. This hypothesis of a fetal origin of adult diseases as initially proposed by Barker et al. (7) has been reinforced by epidemiological evidence of an inverse correlation between birth weight and the risk for cardiovascular disease (17, 23, 30). Cardiovascular system regulation strongly depends on sympathetic autonomic control. Sympathetic activity is believed to play an important role in the pathogenesis of essential hypertension (15, 32, 33). During intrauterine life, malfunction of the placenta is the major cause of undernutrition of the developing fetus. The fetus survives by adaptation of metabolic and cardiovascular systems mediated in part by the activation of the sympathetic component of the autonomous system (38). Thus, growth-restricted fetuses show increased levels of catecholamines and glucocorticoids (16, 63). These adaptive events occur at a developmental time when major regulating systems of the organism are believed to still contain flexible set points.

In low-birth-weight adults, surrogates for altered autonomic cardiovascular control, such as an increased pulse rate (18, 45) and, more specifically, altered blood pressure and heart period variability (28, 60), have been described. Several animal models support the connection of an intrauterine adverse environment and alterations of the sympathoadrenergic system (26, 37, 48). Cardiovascular disease is associated with alterations in the activation of the sympathoadrenergic system in the adult (12, 13). Thus, the cardiac autonomic system balance may be permanently altered according to the concept of fetal programming. During the early postnatal period, one may speculate that temporary upregulated systems normalize in response to a normal postnatal environment, whereas permanently altered systems may become apparent, making this time important for analysis.

Heart rate (HR) variability (HRV) is a well-established noninvasive measure of cardiac autonomic control (22, 29). The aim of this study was to analyze the cardiac autonomic balance in small for gestational age (SGA) newborns by HRV measurements. These electrophysiological findings were supplemented by salivary α-amylase measurements in response to a stress stimulus. Salivary α-amylase has been suggested to be a surrogate for cardiovascular autonomic system balance correlating well with HRV parameters (10, 41), thereby making this parameter a promising indicator for cardiac autonomic function.

METHODS

The study was approved by the Research Ethics Committee of the University of Zurich and the Federal Ethics Commission of the canton of Zurich. Written maternal consent was obtained. SGA was defined as newborn weight below the fifth percentile of the gender-specific newborn reference chart (58). Newborn weights of >10th and <90th percentiles were required for appropriate for gestational age (AGA) infants. Only healthy newborns delivered after 34 wk of gestation (≥238 days postmenstruation) without intensive care requirements, invasive procedures, or malformations were included. Since mothers did not always give consent for both ECG and saliva sample collection, two separate populations had to be analyzed.

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HRV Measurements

A total of 54 children was recruited for 24-h Holter ECG measurements containing 27 AGA and 27 SGA neonates. Gestational age at delivery did not differ between groups ($P = 0.27$). A summary of newborn data is shown in Table 1. Three-channel Holter monitors (Lifecard, Delmar Reynolds Medical, Hertford, UK) were placed within the fourth postnatal day. Ectopic beats, noisy data, and artifacts were manually identified and excluded from the HRV analysis. For the calculation of HRV parameters, HRV Analysis software (version 9.3.0) from Nevrokard (www.nevrokard.eu) was applied.

According to the recommendations of the Task Force of the European Society of Cardiology and North American Society of Pacing and Electrophysiology (55) and the literature for neonatal HRV measurements (5, 19, 47, 57), the following parameters were analyzed.

Time-domain parameters. Time-domain parameters included the following: *1*) as an estimate of overall HRV, the SD of all valid NN intervals (SDNN); *2*) parameters mostly influenced by parasympathetic activity, including the ratio of the number of all pairs of adjacent NN intervals differing by >50 ms and the total number of RR intervals (s-NN50), those differing by >27 ms and the total number of RR intervals (s-NN27), and those differing by >20 ms and the total number of RR intervals (s-NN20) as well as the average of the hourly square root of the mean of the sum of the squares of differences between adjacent NN intervals (r-MSSD); and *3*) parameters mostly influenced by sympathetic activity, including the SD of the average of valid NN intervals (SDANN) in 5-min segments in the recording and the average of the hourly means of SDs of all NN intervals (SDNNi) in 5-min segments. As a geometric index, the HRV triangular index, defined by the total number of all NN intervals divided by the height of the histogram of all NN intervals measured on a discrete scale with bins of 7.8125 ms as an estimate of overall HRV, was calculated.

Frequency-domain parameters. Power spectral analysis was calculated by fast Fourier transformation (Hamming window) in four frequency bands. To account for the neonatal physiology, frequency bandwidths were adjusted according to the literature (19, 47): high frequency (HF) was 0.24–1.04 Hz, representing parasympathetic activity; low frequency (LF) was 0.04–0.24 Hz, representing both sympathetic and parasympathetic activity; very LF (VLF) was 0.003–0.04 Hz, mainly resulting from parasympathetic activity; and ultra LF (ULF) was 0.000–0.003 Hz. Total power (in ms^2) as well as the ratio of LF to HF power (LF/HF), considered as a marker of sympathetic-parasympathetic system balance, were analyzed.

α-Amylase Measurements

Salivary α-amylase levels of 36 AGA and 18 SGA infants were analyzed. The infant's age at delivery did not differ between groups ($P = 0.8$). A summary of newborn data is shown in Table 2.

To analyze sympathetic autonomic nervous system activity and reactivity to stress, salivary α-amylase samples were collected using a routinely performed blood sampling (heel prick test) 72–96 h postpartum as a pain-induced stress factor. This procedure has been shown to be a significant stressor for the newborn (31, 34), and salivary α-amylase has been suggested as a measure of endogenous adrenergic activity and changes in the autonomic nervous system in general (11, 41) and specifically for cardiac autonomic balance (10, 21, 41). Saliva samples were collected from each infant 10 min before and 5 and 20 min after stress induction. Collection time was based on experiments revealing peak α-amylase responses between 5 and 10 min after stress induction (41, 42). A cotton swab was placed in the neonate's mouth for a collection time of 5 min. Samples were placed in saliva collection tubes (Salivette, Sarstedt, Nümbrecht, Germany) and stored frozen at −20°C until further analysis.

Table 1. *Infant baseline characteristics for heart rate variability measurements*

	AGA	SGA	*P* Value
Gestational age, days	270 (239–293)	261 (240–283)	0.272
Birth weight, g	3,170 (2,000–3,830)	2,210 (1,340–2,760)	<0.001
Weight percentile	54.8 (15.2–82.6)	1.0 (0.1–4.2)	<0.001
Gender, male/female	7/20	12/15	0.154

Values are medians with ranges in parentheses; $n = 27$ appropriate for gestational age (AGA) neonates and 27 small for gestational age (SGA) neonates.

Table 2. *Infant basic characteristics for α-amylase measurements*

	AGA	SGA	*P* Value
Gestational age, days	271 (240–294)	266 (240–292)	0.802
Birth weight, g	3,245 (2,100–3,740)	2,265 (1,470–2,870)	<0.001
Weight percentile	48.2 (15.4–91.6)	1.6 (0.1–4.3)	<0.001
Gender, male/female	16/18	6/12	0.341

Values are medians with ranges in parentheses; $n = 34$ AGA neonates and 18 SGA neonates.

We used the amylase 4,6-ethylidene-*p*-nitrophenyl-α,D-maltoheptaoside method from Roche Diagnostics (Mannheim, Germany) for the measurement of α-amylase concentrations in saliva. The diluted saliva samples (1 + 9) were analyzed with integra system 800. The assays showed good performance characteristics (intra-assay coefficients of variation of <1.0% and interassay coefficients of variation of ≤1.3% at concentrations of 79.9 and 198 U/l).

All statistical analyses were performed with STATA 9 statistics/data analysis software (Stata, College Station, TX) according to Altman's and Matthews et al.' recommendations (3, 36). Baseline characteristics of SGA and AGA infants were compared using the Mann-Whitney test and χ^2-test when appropriate. Since HRV parameters were not normally distributed as analyzed by the Shapiro-Francia *W*-test, we compared SGA and AGA values using the Mann-Whitney test. A stepwise multiple regression was conducted to analyze the impact of gender, gestational age, birth weight independent of gestational age, and mode of delivery (spontaneous vaginal, operative vaginal, and cesarean section). α-Amylase data were log transformed to normalize the distribution. The difference between α-amylase baseline levels and the time point of "5 min post" was calculated, and differences revealed no deviation from a normal distribution ($P = 0.49$, Shapiro-Francia *W*-test). A paired Student's *t*-test for unequal samples was used to analyze alterations of log-transformed data between study groups. The Mann-Whitney test was used for the comparison of raw baseline α-amylase levels. Stepwise multiple regression was applied to test for putative influencing factors of α-amylase values such as gestational age, birth weight independent of gestational age, mode of delivery (spontaneous vaginal, operative vaginal, and cesarean section), and gender. The level of statistical significance of all analyses was set at $P < 0.05$.

RESULTS

Infant HRV

Median birth weight of SGA infants was 2,210 g, corresponding to the 1.0st weight percentile, compared with 3,170 g, corresponding to the 53rd percentile in AGA infants. The median gestational age in both groups was comparable (261 vs. 270 days, $P = 0.27$). A summary of study population characteristics is shown in Table 1.

Time-domain parameters as an estimate of overall HRV, such as SDNN and the triangular index, were not significantly

different in SGA neonates compared with AGA neonates ($P = 0.14$ and $P = 0.29$, respectively). The same was found for parameters mostly influenced by sympathetic activity, such as SDANN and SDNNi ($P = 0.34$ and $P = 0.22$, respectively). Again, parameters mostly influenced by the vagal tone, such as r-MSSD ($P = 0.99$) as well as the proportion of pairs of adjacent NN intervals differing by >50, >27, and >20 ms ($P = 0.93$, $P = 0.98$, and $P = 0.91$, respectively), revealed no significant differences between SGA and AGA neonates (Table 3). Analysis of all frequency-domain parameters again revealed no significant differences in SGA infants. Thus, HF, representing vagal activity ($P = 0.82$), LF, representing both sympathetic and vagal activity ($P = 0.82$), VLF ($P = 0.05$), ULF ($P = 0.06$), total power ($P = 0.06$), and LF/HF, representing sympathovagal balance ($P = 0.62$), were comparable in SGA and AGA infants (Table 3). LF/HF similarly decreased with advancing gestational age in both SGA and AGA infants (not shown). To test for a putative influence of gestational age, gender, birth weight independent of gestational age, and mode of delivery on HRV parameters, a stepwise multiple regression was conducted, revealing an influence of gestational age ($P = 0.02$) and gender ($P = 0.02$) but not of birth weight ($P = 0.95$) or mode of delivery ($P = 0.34$) for LF/HF. For other time- and frequency-domain parameters, only gestational age remained as an influencing factor, but not gender.

Infant α-Amylase Levels

The median birth weight of SGA children was 2,265 g, corresponding to the 1.6th weight percentile, compared with 3,425 g, corresponding to the 48.2th percentile in AGA children. A summary of the characteristics of the study population is shown in Table 2.

Median values for baseline α-amylase levels were slightly lower but not significantly different in SGA neonates compared with AGA neonates ($P = 0.13$; Fig. 1). After the application of the stress stimulus, α-amylase levels both slightly increased in AGA and SGA neonates at the time point of "5 min post," not revealing statistically significant differences ($P = 0.3$; Fig. 2).

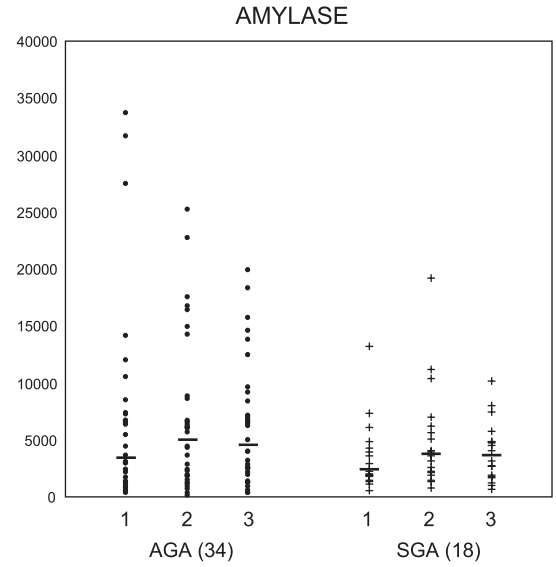

Fig. 1. Salivary absolute levels (U/l) and medians for α-amylase levels of appropriate for gestational age (AGA; $n = 34$) newborns and small for gestational age (SGA; $n = 18$) newborns before (baseline = 1) and after (5 min post = 2; 20 min post = 3) application of the stress stimulus.

To control for an influence of mode of delivery, gender, gestational age, and birth weight independent of gestational age, a stepwise multiple regression was performed, revealing no significant influence ($P = 0.81$, $P = 0.16$, $P = 0.22$, and $P = 0.47$, respectively).

DISCUSSION

We have shown that indicators of cardiac autonomic nervous activity, such as HRV, are preserved in SGA neonates and that the stress-induced α-amylase response is not significantly altered. Thus, our results suggest normal sympathoadrenergic cardiovascular activity in SGA neonates.

Table 3. *Time- and frequency-domain heart rate variability parameters in AGA and SGA infants*

	Median		Minimum		Maximum		
	AGA	SGA	AGA	SGA	AGA	SGA	*P* Value
Heart rate, beats/min	131	130	112	107	154	149	0.29
Mean NN, ms	457.9	461.9	395.5	402.74	538.2	561.98	0.23
Triangular index	20	17	8	7	34	38	0.29
s-NN50, %	2.11	2.42	0.14	0.11	11.56	16.29	0.93
s-NN27, %	10.84	10.36	1.71	0.81	29.92	33.41	0.98
s-NN20, %	20.14	20.1	4.61	2.89	42.4	44.48	0.91
SDNN, ms	63.83	57.45	31.88	29.93	108.4	117.4	0.14
SDNNi, ms	37.33	35.39	18.79	18.63	61.38	72.85	0.22
r-MSSD, ms	19.26	20.52	10.54	9.16	36.27	45.64	0.99
SDANN, ms	48.03	43.23	24.11	21.59	85.18	92.07	0.34
HF, ms^2	0.52	0.56	0.16	0.11	2.36	2.5	0.82
LF, ms^2	2.09	2.1	0.59	0.35	5.73	9.6	0.82
VLF, ms^2	4.77	3.34	0.98	0.88	10.11	13.05	0.052
ULF, ms^2	6.91	4.34	1.26	1.56	26.13	22.12	0.06
Total power, ms^2	13.75	11.08	2.99	3.85	42.8	47.04	0.06
LF/HF	3.75	3.53	1.69	1.92	9.05	8.59	0.62

s-NN50, ratio of the number of all pairs of adjacent NN intervals differing by >50 ms and the total number of RR intervals; s-NN27, ratio of the number of all pairs of adjacent NN intervals differing by >27 ms and the total number of RR intervals; s-NN20, ratio of the number of all pairs of adjacent NN intervals differing by >20 ms and the total number of RR intervals; SDNN, SD of all valid NN interval; SDNNi, average of the hourly means of SDs of all NN intervals; r-MSSD, average of the hourly square root of the mean of the sum of the squares of differences between adjacent NN intervals; SDANN, SD of the average of valid NN intervals; HF, high frequency; LF, low frequency; VLF, very LF; ULF, ultra LF.

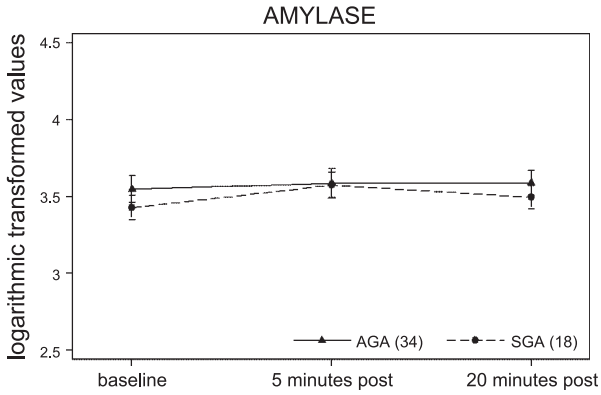

Fig. 2. Log-transformed salivary levels for α-amylase during resting conditions and after stress induction in AGA ($n = 34$) and SGA ($n = 18$) neonates. Values are means ± SE. Maximal α-amylase response levels were not significantly different between AGA and SGA neonates ($P = 0.3$).

There is evidence from animal studies that an unfavorable prenatal environment may alter the sympathetic autonomic system balance, which seems to persist into postnatal life. As such, prenatal stress has been shown to result in increased basal and stimulated circulating catecholamine levels in adult rats (26, 61), and compromised intrauterine blood flow did result in a downregulation of epinephrine levels in sheep (1, 43, 51). Conversely, chronic prenatal hypoxia has been shown to be associated with sympathetic hyperinnervation (48, 49). Thus, chronic intrauterine hypoxemia may suppress the adrenaline synthetic capacity of the adrenal medulla (37, 43, 51), whereas increased circulating norepinephrine concentrations seem to derive from hyperinnervated sympathetic nerve terminals of fetal vessels and tissues (37).

More specifically for the cardiovascular system, growth-restricted newborn rats displayed significantly increased basal cardiac sympathetic neuronal activity, as determined by [^{3}H]norepinephrine tracer and α-methyltyrosine techniques (51). In the same species, intrauterine exposure to exogenous steroid levels produced persistent abnormalities of cardiac noradrenergic innervation (8).

In the human, Flanagan et al. (18) found an inverse relationship between adult resting pulse rate as an index related to sympathetic activity and birth weight. Recently, markers of impaired fetal growth were related to autonomic cardiovascular control in adults involving modulation of both sympathetic and parasympathetic function. Accordingly, blood pressure, HR, its spectrum analysis, and baroreflex sensitivity in response to psychological stress were found to be altered in low-birth-weight women but not in men of an Australian prospective cohort study (28, 59). Cardiac sympathetic nerve activity as analyzed by cardiac preejection period and respiratory sinus arrhythmia has been shown to be increased in adolescents born growth restricted (24). In contrast, inconsistent results on muscle vascular bed sympathetic nerve traffic have been reported in low-birth-weight adults (9, 62).

HRV is a well-established noninvasive measure of cardiac autonomic control that has been shown to be related to hypertension (22, 29) and to predict future adverse cardiovascular events in adults (2) and has been suggested for putative prognostic use in children (54). While blood pressure as a cardiovascular risk factor has been shown to be inversely related to birth weight in adults, to our knowledge, a systematic analysis of HRV parameters according to stardardized methods (55) so far has not been performed in adults with low birth weight. Alterations of HRV in adults, however, may not necessarily represent a prenatal initiated event according to the concept of fetal programming considering the close interaction with other neuroendocrine systems such as the hypothalamus-pituitary-adrenal (HPA) axis (8).

We did not find significant alterations of HRV parameters in SGA neonates. These findings are consistent with the study of Mehta et al. (39), who found no significant correlation between various HRV parameters and birth weight in a population of 96 healthy newborns, although these authors may not have applied appropriate frequency bands for the frequency-domain analyses (19). In contrast, a small study by Spassov et al. (52) described significantly decreased HRV parameters in term SGA newborns during sleep at 2–10 days of postnatal life and found significantly shorter NN intervals in SGA neonates, which we did not observe despite similar SGA criteria. The reason for these differing results are not clear but may be explained by the small sample size and the different experimental setting of that study. Indeed, it has been shown that HR increases steeply after the 5th day of life with a maximum on the 10th day, indicating distinct changes in cardiovascular control (40). Therefore, it cannot be excluded that group differences in postnatal age may have had an important influence on these results. Furthermore, single HRV parameters could not be compared directly since long-term recordings for time- and frequency-domain parameters were calculated in our study as opposed to the short-term frequency-domain recordings in the study of Spassov et al. (52). Nevertheless, these parameters seem to correlate well (39, 55). Galland et al. (20) did not observe significant differences in NN intervals in SGA infants, confirming our data. They found an increased resting sympathetic tone using Poincaré plot data analysis, however, lying at the limits of significance ($P = 0.046$) only after controlling for HR at 1 and 3 mo of age and attributed these findings to an immaturity of the autonomic nervous system. Studies on functional central nervous system maturation in SGA infants, however, have revealed conflicting results (4, 27, 44). There is evidence that HRV correlates with birth weight in 11- to 12-wk-old infants but not in younger infants (35). These data suggest alterations of the cardiac autonomic nervous system rather beyond the neonatal period. Therefore, one tends to speculate that the cardiac autonomic nervous system rather appears to be vulnerable during the postnatal development due to conditions induced by putative permanently altered regulatory systems such as the HPA axis (46) and influence of postnatal catchup growth (35). In support of this notion, it has been shown in human fetuses that antenatal glucocorticoids transiently lower short- and long-term fetal HRV (14). Alternatively, we cannot exclude that prenatal induced alterations are too small to detect in the neonatal period and only become apparent with increasing system maturation postnatally. Even more, different methods thought to represent cardiac autonomic activity may have different selectivity for sympathetic system subunits (25), and different methods of stress induction may produce different results (28).

Statistical analysis of our HRV parameters revealed three frequency-domain parameters (total power, VLF, and ULF) to be close to the level of statistical significance (Table 3). These

parameters are closely connected as VLF and ULF components correspond to up to 95% of the total power. Putative reductions in total power as an estimate for autonomic nervous system global activity and VLF might indicate a decrease in parasympathetic modulation. The physiological correlate of VLF and ULF, however, is not truly well established (55). Therefore, although a false negative result cannot be completely excluded for these parameters and a definitive conclusion may not be drawn due to a relatively small sample size, all remaining frequency- and time-domain parameters did not suggest statistical significant differences of HRV in SGA neonates.

Direct comparison of these components with results from the literature is rather difficult due to different experimental settings and HRV calculations; however, Galland et al. (20) reported a higher resting sympathetic tone analyzing 23 SGA infants using a Poincaré method for standard deviation of the beat intervals (SDRR) and the standard deviation of the change between successive beat intervals (SDΔRR). However, only the SDRR/SDΔRR ratio reached the limit of statistical significance ($P = 0.046$), whereas the remaining parameters were not statistically different. In the study of Spassov et al. (52), short-term HRV with different frequency bands in 10 SGA infants were analyzed; however, not all frequency parameters (LF) reached statistical difference, and HR was significantly increased in SGA neonates, potentially contributing to these findings. In contrast, Galland et al.'s and our analysis did not observe increased HR in SGA neonates.

There is evidence that autonomic cardiovascular control and size at birth may be gender specific. Accordingly, adult low-birth-weight women showed altered autonomic and baroreflex parameters in response to psychological stressors, but not men (28, 60). In our study, which conducted a stepwise multiple regression, we found an influence of gender when analyzing the entire data only for LF/HF but not for the remaining time and frequency domains, making a gender effect at this time of development rather questionable. However, gender was unequally distributed between groups, and subgroups were comparatively small; therefore, a definitive conclusion may not be drawn. It is possible, however, that gender-specific hormonal influences may manifest during adolescence and adulthood in SGA infants.

To supplement our electrophysiological findings with a neuroendocrinological approach, we analyzed salivary α-amylase levels during resting conditions and after a stressful stimulus. Acinar cells in the salivary glands are richly innervated by both sympathetic and parasympathetic nerve fibers, influencing the release of salivary α-amylase by classic neurotransmitters (56). Studies in humans and animals have suggested that the activation of the autonomic nervous system leads to a high activity of salivary α-amylase (6, 11, 50, 53). Furthermore, α-amylase levels have been found to be associated with cardiovascular physiology and are suggested to be a surrogate for cardiovascular autonomic system balance (21). Bosch et al. (10) found a significant negative correlation between the parasympathetic-influenced HRV parameter r-MSSD and α-amylase levels during stress induction in adults. Furthermore, a positive correlation between α-amylase levels and LF/HF as a surrogate for sympathetic tone has been shown (41), thereby making this parameter a promising indicator for cardiac autonomic function.

To our knowledge, α-amylase levels in SGA neonates have not been studied before. Although studies on cardiovascular autonomic physiology using α-amylase measurements have not been validated in neonates, several analyses from children support the strong relationship between salivary α-amylase and sympathetic/parasympathetic nervous system activation in younger individuals (21). The comparable α-amylase levels during resting conditions in AGA and SGA neonates in our study and the absence of significant differences in response to stress induction support the notion that the sympathetic autonomic cardiovascular system seems not to be permanently altered postnatally regarding the fetal origin hypothesis. The sympathetic nervous system, however, is composed of multiple function-specific subunits (25), and programming of sympathetic nervous system function is believed to occur regionally rather than on a global basis, suggesting that subdivisions of the sympathetic nervous system may be influenced by different sets of environmental variables (64). Therefore, we cannot exclude that other subunits of the sympathetic nervous system may be permanently altered in SGA neonates. Furthermore, with regard to HRV techniques as well as α-amylase measurements, although in general use as research tools for the assessment of cardiac autonomic activity, it is not entirely clear to what degree they truly represent autonomic activity in neonates.

In conclusion, HRV and salivary α-amylase levels, as indicators of cardiac autonomic activity, are not altered in SGA neonates compared with AGA neonates. Thus, the neonatal sympathovagal balance appears to be preserved in SGA neonates.

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Antenatal betamethasone administration alters stress physiology in healthy neonates.

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Antenatal Betamethasone Administration Alters Stress Physiology in Healthy Neonates

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OBJECTIVE: To analyze hypothalamic-pituitary-adrenal axis balance in healthy newborns after antenatal betamethasone treatment for lung maturation where delivery could be prolonged until or near term.

METHODS: In a prospective observational study, salivary cortisol and cortisone levels were measured at the fourth day of life during resting conditions and in response to a pain-induced stress event in 23 neonates with antenatal exposure to a single course of betamethasone (2×12 mg) and compared with 40 controls. The mean interval between betamethasone treatment and delivery was 60±23 days.

RESULTS: On day 4 of life, neonates in the control group exhibited a significant increase in cortisol and cortisone from baseline levels after the stress induction (1.175–2.4 ng/mL for cortisol and 11.35–18.15 ng/mL for cortisone [both $P<.05$]), whereas, in betamethasone-exposed neonates, cortisol and cortisone stress response was not significantly different from baseline levels (1.39–1.6 ng/mL for cortisone [$P=.76$] and 14.8–17.1 ng/mL for cortisol [$P=.69$]). No influence of gestational age at betamethasone administration ($P=.76$) or gestational age at delivery ($P=.71$) on stress response patterns was observed in a multiple stepwise regression.

CONCLUSION: A single course of antenatal betamethasone treatment induces a suppression of stress reactivity in healthy newborns.

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LEVEL OF EVIDENCE: II

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Antenatal betamethasone administration in pregnancies at risk for preterm delivery before 34 weeks of gestation is an established and effective procedure to improve neonatal outcome by decreasing neonatal mortality and infant morbidity.[1] However, in different animal models, excessive prenatal glucocorticoid administration reduces birth weight and increases blood pressure and glucose levels and alters the activity of the hypothalamic–pituitary–adrenal axis in adults.[2] Although long-term studies in the human after antenatal corticosteroid treatment have not found adverse effects on general health and cardiovascular risk factors at 20 and 30 years, there was evidence for insulin resistance in these individuals[3,4] and clinically apparent symptoms for cardiovascular disease may even appear at older ages. Moreover, hypothalamic–pituitary–adrenal axis regulation has not been assessed in these studies.

Therefore, concerns have been raised as to whether prenatal treatment with corticosteroids may entail long-term consequences for the health of these individuals. The hypothalamic–pituitary–adrenal axis, which is considered to be one of the major systems involved in the fetal origin of adult diseases hypothesis, has been shown to be vulnerable to excess glucocorticoid exposure during its maturational stage.[5,6] Endogenous glucocorticoids are involved in mediation of developmental steps of endocrine and neurohumoral systems,[7,8] and interference with this fine neuroendocrine balance may alter the function of these systems permanently.

Studies in premature neonates have shown that a single course of antenatal betamethasone causes suppression of stress reactivity because these neonates failed to increase cortisol levels in response to a stressor.[9,10] Prematurity per se, prematurity-associated morbidity, and the stressful environment of a neonatal intensive care unit (NICU) may have a considerable effect on neonatal stress regulation.[11] Thus, allocation of these effects to a specific event such as

antenatal betamethasone treatment appears difficult, especially when considering that a pregnancy-associated complication usually precedes preterm delivery.

We therefore analyzed the hypothalamic–pituitary–adrenal axis reactivity of healthy, late preterm or term newborns who had received a single course of antenatal betamethasone treatment for imminent preterm delivery before 34 weeks of gestation and in whom preterm delivery was postponed for more than a mean of 8 weeks. We hypothesized that stress reactivity in these newborns is altered in comparison with those in the control group.

MATERIALS AND METHODS

The study was approved by the Research Ethics Committee of the University of Zurich and the federal ethics commission of the canton of Zurich, and written maternal consent was obtained from all participants in the study.

Only healthy newborns delivered after 34 weeks of gestation (more than 238 days) were included in the study. Forty-four neonates who had received antenatal betamethasone administration (2×12 mg within 24 hours) for imminent preterm delivery were delivered after 34 weeks of gestation during the study period (September 2006 to April 2007). In 21 cases, either parents refused to participate in the study (16 cases) or saliva collection was inadequate (five cases). Saliva samples of 23 neonates (study group) could be included in our analysis. The experience from a previous study allowed us to presume that, with regard to the variability of cortisol levels, approximately half of the number of controls provides a statistically adequate study group sample size. Accordingly, 40 healthy neonates, whose cases have been published previously,[12] served as controls (control group). These neonates were born in the same institution between April and September 2005, and methods for saliva collection and laboratory analysis were identical. Neonatal weight between the 10th and 90th percentiles was required for both study groups. Newborns were excluded from analysis when intensive care treatment or invasive procedures had been necessary, when malformations were present, or when insufficient amounts of saliva were collected. Furthermore, maternal substance abuse (nicotine, alcohol) during pregnancy was an exclusion criterion. No signs of clinically apparent infections were present in either group. Two pregnancies in the study group were complicated by preterm premature rupture of membranes and four pregnancies by vaginal bleeding, leading to betamethasone treatment for imminent preterm delivery. The remaining cases were treated for preterm contraction and cervical ripening. Ultrasonographic measurements of fetal crown-rump length and biparietal diameter during the first trimester served as parameters for accurate determination of gestational age.

Stress reactivity was tested in newborns using a standardized procedure with an automated heel lance (heel-prick test) as a stress event. This test is a routinely applied screening test in which blood is sampled and analyzed for metabolic disorders (Guthrie test). Saliva samples were collected from each neonate 10 minutes before and 20 minutes after the stress event 72–96 hours postpartum between 8 AM and 1 PM and analyzed for salivary cortisol and cortisone levels. Baseline levels were obtained in an undisturbed environment. Collection time was based on experiments revealing peak cortisol responses between 20 and 30 minutes postmanipulation.[13] In detail, a cotton swab was placed in the neonate's mouth for a collection time of 5 minutes. Salivary cortisol reflects the unbound, active fraction of cortisol and is highly correlated with plasma cortisol levels.[14,15] Samples were placed in saliva-collection tubes (Salivette, Sarstedt, Nümbrecht, Germany), immediately frozen, and stored at -20°C until further analysis.

Saliva cortisol and cortisone were determined simultaneously by liquid chromatography tandem mass spectrometry, with atmospheric pressure chemical ionization in the positive ion mode, according to a modified method of Rauh et al[16] using 100 microliters of the samples and calibrators, as previously described in detail.[12]

We applied STATA 9 statistics/data analysis software (Stata Corporation, College Station, TX) for statistical analysis according to Altman's recommendations for repeated measurements that rise from the same individuals studied under different circumstances.[17] Baseline characteristics were compared using the Mann-Whitney test and χ^2 test when appropriate. Because basal levels of cortisol vary considerably in individual newborns and children,[13] absolute values and median relative alterations are presented. Because cortisol and cortisone values were not distributed normally as analyzed by the Shapiro-Francia W' test, we analyzed the difference between cortisol and cortisone baseline levels and time point "20 minutes post" using the Wilcoxon signed rank test. Mann-Whitney test was used for comparison of baseline cortisol and cortisone levels. To account for within-subject variations, a two-way analysis of variance for repeated measurements was conducted. A stepwise multiple regression was applied to test for putative

influencing factors on cortisol alterations such as gestational age at betamethasone administration, gestational age at delivery, neonatal weight, and sex. The resulting regression coefficient indicated the predicted increase in the dependent variable for each unit increase in the explanatory variable. To validate our statistical analyses, a power calculation was conducted for the given sample size (n=23), assuming the mean increase in cortisol levels of neonates in the control group as relevant. Finally, cortisol and cortisone levels in the study group were tested for correlation using the Spearman rank correlation test. The level of statistical significance of all analyses was set at $P<.05$.

RESULTS

The mean interval between betamethasone administration and birth was 60 (+23) days. Gestational age, birth weight, weight percentile, and head circumference at birth did not differ significantly between the study group and control group. Betamethasone was administered at a mean gestational age of 29.4 (+2.6) weeks. A summary of the characteristics of the study population is given in Table 1.

The median baseline levels for cortisol and cortisone did not differ significantly between neonates in the study group and those in the control group (1.39 ng/mL [range 0.09–9.82] compared with 1.175 ng/mL [range 0.09–15.7] for cortisol, $P=.42$; 14.8 ng/mL [range 2.6–36] compared with 11.35 ng/mL [range 5.83–44.3] for cortisone, $P=.34$, respectively). Individual baseline and stimulated cortisol and cortisone levels are indicated in Figure 1.

In neonates in the control group, analysis of the physiological stress response revealed a significant increase in cortisol and cortisone levels in response to the heelstick, as was expected (2.4 ng/mL [range 0.3–12.2], 18.15 ng/mL [range 2.8–43.1], respectively, $P<.05$, Fig. 1). In contrast, neonates in the study group displayed a noticeably blunted stress response—cortisol and cortisone release was not significantly different from baseline levels (1.6 ng/mL [range 0.2–11.3], $P=.76$ and 17.1 ng/mL [range 7–32.9], $P=.69$, respectively, Fig. 1). A two-way analysis of variance again revealed that there was no significant alteration of cortisol after the stress stimulus in the study group ($P=.65$) as opposed to control group ($P=.003$). A one-sample *t*-test power calculation validated the absent cortisol response in neonates in the study group with a power of 99% at a significance level of 0.05. For better visualization of alterations before and after stress stimulus medians of relative alterations for cortisol and cortisone, see Figure 2.

To test whether the reduced cortisol response in the neonates in the study group could be influenced by gestational age at steroid administration, a stepwise multiple regression model was applied, revealing no significant association ($P=.76$). Subsequently, neonatal weight, sex, and gestational age at delivery were tested, and none of them had a significant influence on cortisol response levels in the study group ($P=.71$, $P=.74$, $P=.71$, respectively) (Table 2). Finally, we tested whether the decreased cortisol response in the study group was mediated by an increased conversion of cortisol to cortisone. Cortisol and cortisone levels were strongly correlated in the study group, thus excluding this possibility ($P=.76$).

DISCUSSION

Our results indicate that a single course of betamethasone administration in pregnancies at risk for preterm delivery entails alterations in hypothalamic–pituitary–adrenal axis responsiveness that persist into

Table 1. Maternal and Neonatal Baseline Characteristics

	Control (n=40)	Study Group (n=23)	*P*
Gestational age (d)	273 (240–294)	266 (242–284)	.201
Birth weight (g)	3,288 (2,100–3,780)	2,950 (2,220–3,930)	.269
Weight percentile	52 (17.6–91.6)	39 (11.9–87.3)	.617
Male/female	18/22	13/10	.907
Head circumference	34.5 (32.5–36)	34 (32–37)	.583
5-min Apgar score	9 (8–10)	9 (8–9)	.517
Maternal hospital stay (d)	0 (0–11)	8 (0–85)	<.001
Maternal age (y)	30 (18–39)	29 (23–37)	.496
Parity	2 (1–4)	1 (1–3)	.040
Maternal pregestational BMI	23 (16–38.6)	21.3 (18.3–36.9)	.100
Maternal BMI at delivery	27.7 (23.7–41.9)	26.2 (21.8–39)	.153

BMI, body mass index.
Data are median (range) unless otherwise specified.

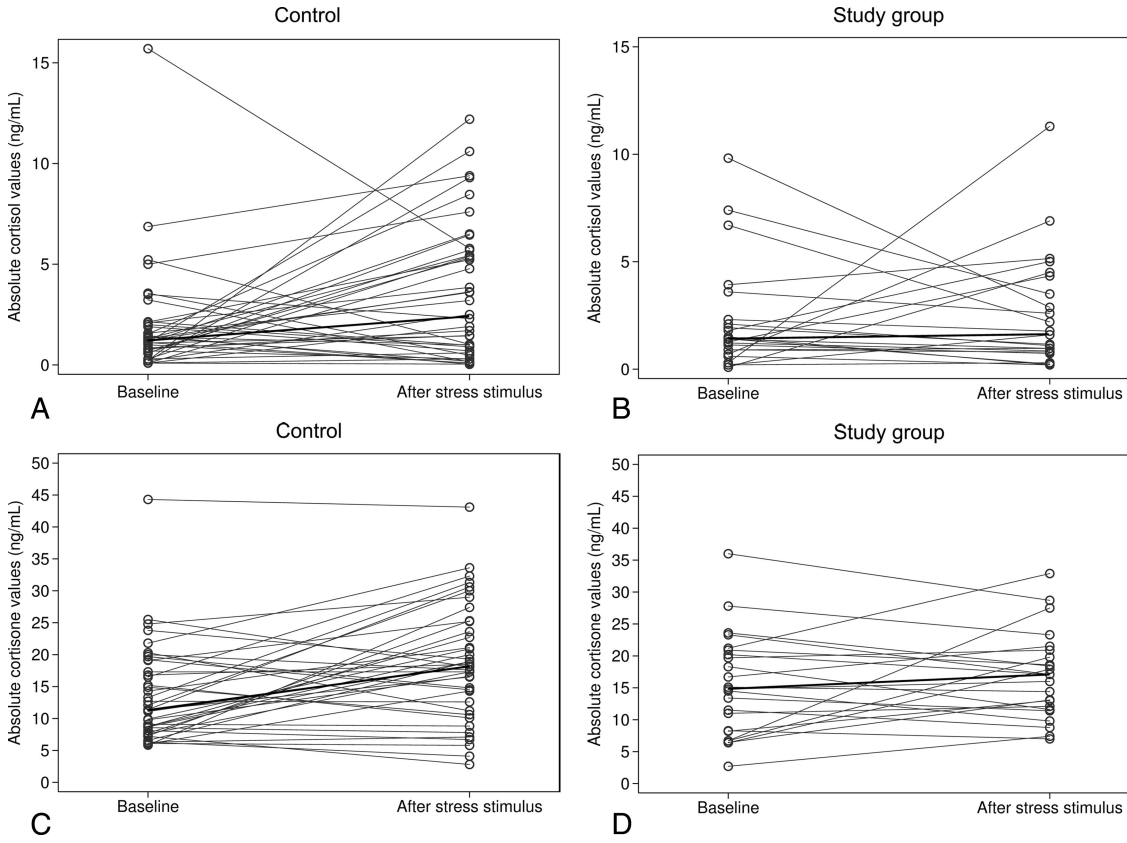

Fig. 1. Salivary absolute levels for cortisol **(A, B)** and cortisone **(C, D)**, including individual courses of control (n=40) and study group (n=23) newborns before (baseline) and after (after stress stimulus) application of the stress stimulus. The *line* indicates median levels.

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postnatal life, even if delivery can be avoided until or near term.

Previous studies in premature neonates (28–30 weeks of gestation) have suggested that antenatal betamethasone treatment persistently suppresses cortisol responsiveness to a stressor for at least 4–6 weeks after birth.[10] Owing to the setting of that study, however, prematurity itself as well as the effect of stressful postnatal intensive care unit handling may have had considerable influence on these results. Unfortunately, owing to the lack of an appropriate control group of equal gestational age, these neonates were compared with a control cohort delivered at 33–34 weeks of gestation, significantly later than the study group. Thus, these cofactors could not be controlled for. In a small study population (n=9), however, the same group of investigators analyzed the hypothalamic–pituitary–adrenal response in a NICU situation in preterm neonates born at 33–34 weeks of gestation, again revealing a decrease in cortisol response after antenatal betamethasone administration.[9]

Baseline cortisol levels after antenatal corticosteroid treatment have been shown to be suppressed transiently for 2–7 days in preterm neonates,[18,19] and cortisol response levels to a pharmacologic challenge were lower in preterm very low birth weight neonates after a single course of antenatal betamethasone administration 7 days postpartum. Curiously, this could not be shown for multiple doses of betamethasone administration. However, at 14 days postpartum, these differences no longer could be observed, thus indicating a transient effect.[11] In fact, there is evidence, that premature newborns have an immature hypothalamic–pituitary–adrenal axis so that the hypothalamus may fail to recognize the stimulatory signal or adrenal steroidogenesis may be ineffective yet.[20–22]

The present study is the first to analyze stress physiology in healthy term or near-term neonates after a single course of betamethasone administration at a much earlier time in pregnancy. Consequently, these neonates did not experience the potentially stressful environment of a NICU, and prematurity-

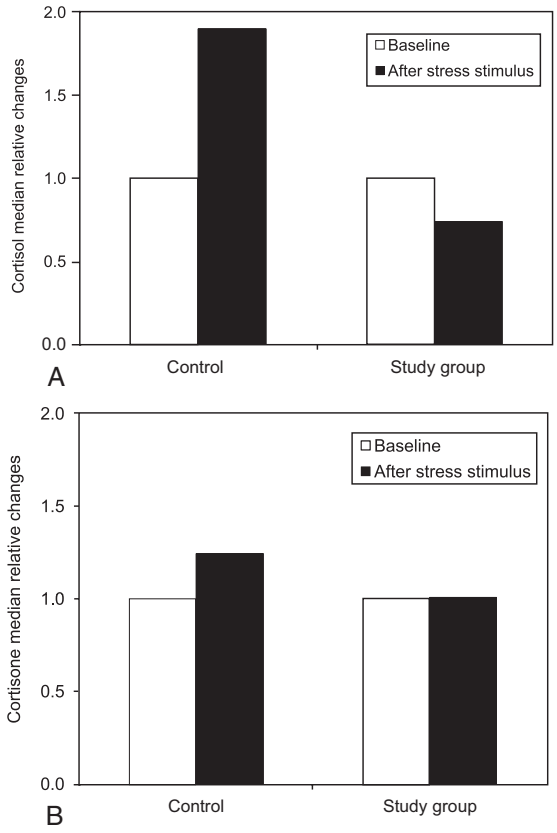

Fig. 2. Median relative salivary level alterations for cortisol after stimulus (1.9 [range 0.02–55.5] compared with 0.72 [range 0.13–50]) **(A)** and cortisone after stimulus (1.2 [range 0.39–4.7] compared with 0.95 [range 0.63–4.1]) for control and study groups respectively **(B)**.

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associated effects with related morbidity can be excluded. Likewise, putative influencing factors such as smoking during pregnancy or intrauterine compromise of the fetus had been excluded explicitly, in contrast to studies in preterm neonates. Furthermore, the mean interval between betamethasone administration and hypothalamic–pituitary–adrenal axis analysis was more than 8 weeks, thereby excluding transient short-term effects.

Table 2. Multiple Stepwise Regression Model

	Regression Coefficient	Standard Error
Gestational age at delivery	0.0721	0.1343
Newborn weight	−0.0013	0.0032
Sex	0.8209	1.9243
Gestational age at time of steroid administration	0.0158	0.0496

Stepwise regression of putative confounding factors on hypothalamic–pituitary–adrenal axis activity. The stepwise backward regression started with this full model; the criterion for removing a variable was $P \geq .200$. Adjusted R^2 for full model −0.1919.

Animal models in different species collectively show that corticosteroid application in pregnancy permanently affects hypothalamic–pituitary–adrenal axis function and behavior in offspring.[5] Unlike endogenous glucocorticoids, synthetic glucocorticoids predominantly bind to glucocorticoid receptors because the mineralocorticoid receptors have low affinity to exogenous glucocorticoids.[23] Thus, the glucocorticoid receptor is likely to be affected predominantly. In the rodent, a single course of antenatal betamethasone induced a decrease in hippocampal glucocorticoid receptor expression.[24] Interestingly, disruption of the glucocorticoid receptor gene in the central nervous system in knock-out mice results in an impaired behavioral response to stress.[25] Constant receptor alterations have been described at the level of the limbic system in the hippocampus (involved in negative feedback), the amygdala (involved in activation), and the paraventricular nucleus.[26–30]

In guinea pigs, a single equivalent dose of glucocorticoids used in pregnant women caused significantly lower hippocampal glucocorticoid receptor mRNA levels in females, accompanied by an attenuated stress response. In contrast, glucocorticoid receptor mRNA levels were elevated in males that showed elevated basal but not stress-induced cortisol levels.[31] Repeated doses of glucocorticoids induced sex-specific differential effects on mineralocorticoid receptor mRNA and glucocorticoid receptor mRNA levels in different regions.[32] Animal models have shown further that females might be more sensitive to hypothalamic–pituitary–adrenal axis programming than males.[33,34] We did not observe a sex-dependent difference in hypothalamic–pituitary–adrenal axis reactivity in our study, just as a study in adults analyzing postmenopausal women.[35] Sex-specific cortisol reactivity in general depends on different levels of corticosteroid-binding globulin and sex steroids.[36] We therefore cannot exclude sex-specific hormonal influences that may manifest at a later time during adolescence and adulthood in these neonates.

The mechanisms underlying hypothalamic–pituitary–adrenal axis balance thus are complex and depend on timing and dose of glucocorticoid exposure.[29] The variability in neurodevelomental profiles of different species further adds to the difficulty in drawing conclusions for the human situation.

Limited data on prenatal glucocorticoid treatment in nonhuman primates indicate structural and morphological alterations in the area of the hip-

pocampus and an altered set point for basal and stress-induced cortisol levels at the age of 9 months.[37] Furthermore, alterations in cytoskeletal proteins and presynaptic terminals involved in neuroplasticity were observed after equivalent doses of betamethasone in the baboon.[38]

In the human, betamethasone-induced alterations in hippocampal mineralocorticoid receptor mRNA expression levels could not be observed in preterm neonates who died 4 days after delivery (n=9); however, glucocorticoid receptor mRNA alterations that may be of major relevance could not be analyzed reliably, which makes it difficult to draw conclusions.[39] Different gestational ages and variabilities in glucocorticoid exposure as well as other factors, such as stress due to intensive care settings, further add to the difficulty of interpreting these data.

The strength of the present study is that study groups are largely free from maternal- and neonatal-derived interfering factors such as prematurity-associated interventions and morbidities. Furthermore, the long interval between betamethasone administration and delivery (mean 60 days) does exclude transient short-term effects of betamethasone on the hypothalamic–pituitary–adrenal axis. Nevertheless, betamethasone administration implies the presence of a risk for preterm delivery, and we cannot exclude the possibility that these risk factors, such as preterm contractions and their treatment, bleeding, undetectable infections, and event- or hospitalization-associated stress situations for the mother, add to the alteration of hypothalamic–pituitary–adrenal axis responsiveness. Indeed, median maternal hospital stay during pregnancy was 8 days. Furthermore, whether our observed effects transform into long-term consequences has to be established by follow-up studies.

We would like to point out that antenatal glucocorticoid administration in pregnancies at risk for preterm delivery has established benefits in reducing neonatal mortality and morbidity,[1] and it is not clear to what extend our observed alterations associated with this procedure really are relevant determinants for diseases in later life. However, if a permanent alteration of the hypothalamic–pituitary–adrenal axis occurs in the human fetus after the application of a single course of betamethasone treatment, hypothalamic–pituitary–adrenal axis function would be expected to be modified already in the neonate. This precondition for a persistent reprogramming of the hypothalamic–pituitary–adrenal axis has been demonstrated in our study. Long-term follow-up studies analyzing hypothalamic–pituitary–adrenal axis reactivity are not yet available. In a 30-year follow-up study of Dalziel et al,[3] basal plasma cortisol levels were unaltered after a single course of maternal betamethasone administration; stress reactivity, however, was not analyzed, and clear indicators for the presence of insulin resistance were present in these individuals.

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Effect of antenatal betamethasone administration on neonatal cardiac autonomic balance.

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Effect of Antenatal Betamethasone Administration on Neonatal Cardiac Autonomic Balance

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ABSTRACT: Beneficial effects of antenatal glucocorticoid treatment in pregnancies at risk for preterm delivery may entail long-term consequences for the establishment of sympathoadrenergic system balance. We analyzed the cardiac autonomic system activity in neonates with a single course of antenatal betamethasone (2×12 mg) treatment by calculating heart rate variability (HRV) time-domain parameters from 24 h ECG recordings and short-term frequency-domain parameters during infant active and resting states. In addition, resting and challenged salivary α-amylase levels were measured in 23 betamethasone-exposed neonates and compared with controls. Indicators for overall HRV (SDNN: $p = 0.258$; triangular index: $p = 0.179$) and sympathovagal balance [low- to high-frequency power (LF/HF): $p = 0.82$ (resting state)] were not significantly different in neonates of the betamethasone treatment group. Parameters mostly influenced by sympathetic activity [SD of the average of valid NN intervals (SDANN): $p = 0.184$ and SDs of all NN intervals (SDNNi): $p = 0.784$] and vagal tone [RMSSD: $p = 1.0$; NN50: $p = 0.852$; HF: $p = 0.785$ (resting state)] were unaltered. Resting α-amylase levels were not significantly different in the betamethasone treatment group ($p = 0.304$); however, α-amylase release after a neonatal challenge was slightly reduced ($p = 0.045$). Thus, cardiac autonomic balance seems to be preserved in neonates exposed to a single course of antenatal betamethasone treatment. **(*Pediatr Res* 68: 286–291, 2010)**

Antenatal glucocorticoid treatment for lung maturation in pregnancies at risk for preterm delivery is an established method to reduce neonatal mortality and morbidity (1). However, there is accumulating evidence that glucocorticoids may alter maturational development and homeostasis of fetal regulatory systems. Repeated antenatal glucocorticoid exposure results in reduced birth weight, increased blood pressure, and glucose levels in adult offspring in different animal models (2).

The cardiac autonomic system has been shown to be sensitive toward antenatal glucocorticoids. Accordingly, fetal heart rate (FHR) variation is altered at least transiently in human fetuses after antenatal glucocorticoids (3,4), and infant heart rate answer to a stress event is significantly increased (5). Putative mechanisms include an altered cardioregulatory innervation and signaling that seem to happen at central levels and at the level of the cardiomyocyte according to rodent models (6,7).

Cardiovascular system regulation strongly depends on sympathetic autonomic control that is believed to play an important role in the pathogenesis of cardiovascular disease in the adult (8,9). Long-term observations in adolescents and young adults with antenatal exposure to glucocorticoids have rather focused on classic signs of disease manifestation such as blood pressure (10,11). However, risk factors for cardiovascular or metabolic disease may transform into apparent clinical manifestation at a more advanced age when stability of these systems can be less compensated by the organism. Therefore, we focused on putative predictive methods characterizing sympathoadrenergic cardiac balance. Heart rate variability (HRV) is a well-established noninvasive measure of cardiac autonomic control (12,13) that is regulated by a complex interplay of sympathetic and parasympathetic branches of the autonomic nervous system. The aim of this study was to analyze the cardiac autonomic balance of healthy newborns at or near term who had received a single course of antenatal betamethasone treatment for imminent preterm delivery before 34 wk of gestation and in whom delivery was delayed for a median of more than 7 wk. Electrophysiological HRV data were supplemented by functional salivary α-amylase measurements in response to a stress stimulus. Salivary α-amylase has been suggested to be a surrogate for cardiovascular autonomic system balance correlating well with HRV parameters (14,15).

METHODS

The study was approved by the Research Ethics Committee of the University of Zurich and conforms with the Declaration of Helsinki. Written parental consent was obtained from all participants.[1]

Healthy infants born after 34 wk of gestation (>238 d postmenstruation) were included in the study. Exclusion criteria were infant intensive care treatment or invasive procedures, malformations, maternal substance abuse (nicotine and alcohol) during pregnancy, or insufficient amounts of saliva collected. No signs of clinical apparent infections were present in both groups. Sonographic measurements of fetal crown-rump length and biparietal diameter during the first trimester served as parameters for accurate determination

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Abbreviations: FHR, fetal heart rate; **HPA,** hypothalamo-pituitary-adrenal; **HRV,** heart rate variability

Table 1. *Neonatal and maternal baseline characteristics for HRV measurements and salivary α-amylase measurements*

	HRV measurements			α-Amylase measurements		
	Control (n = 23)	Treatment group (n = 23)	p	Control (n = 40)	Treatment group (n = 23)	p
Gestational age (d)	275 (239–293)	260 (245–289)	0.0504	273 (240–294)	266 (242–284)	0.201
Birth weight (g)	3260 (2150–3830)	3040 (2170–3750)	0.2104	3288 (2100–3780)	2950 (2220–3930)	0.269
Weight percentile	54.8 (15.4–82.6)	50.0 (15.2–88.9)	0.7334	52 (17.6–91.6)	39 (11.9–87.3)	0.617
Male/female	3/20	12/11	0.011	18/22	13/10	0.907
Head circumference	34 (31–37.5)	34.5 (31–36.5)	0.7402	34.5 (32.5–36)	34 (32–37)	0.583
5-Min APGAR	9 (8–10)	9 (8–10)	0.7571	9 (8–10)	9 (8–9)	0.517
Maternal age (y)	30 (16–41)	30 (21–41)	0.8342	30 (18–39)	29 (23–37)	0.496
Parity	1 (1–3)	1 (1–4)	0.6088	2 (1–4)	1 (1–3)	0.040
Maternal pregestational BMI	21.1 (18.9–31.2)	19.7 (17.7–36.9)	0.1142	23 (16–38.6)	21.3 (18.3–36.9)	0.100
Maternal BMI at delivery	26.0 (22.2–32.8)	24.9 (21.8–39.6)	0.5774	27.7 (23.7–41.9)	26.2 (21.8–39)	0.153
Gestational age at steroid administration (d)		219 (178–238)			204 (173–235)	

Data are median (range) unless otherwise specified.
BMI, body mass index.

of gestational age. Because mothers did not always give consent for both ECG and saliva sample collection, the populations were analyzed separately.

HRV measurements. A cohort of 23 infants with antenatal betamethasone treatment was recruited for 24 h Holter ECG measurements (treatment group). The control group of 23 infants without antenatal betamethasone exposure is part of a cohort previously published (16). A summary of infant and maternal characteristics is shown in Table 1. Three-channel Holter monitors (Lifecard CF; Delmar Reynolds Medical, Hertford, United Kingdom) were placed within the third to fourth postnatal day. Ectopic beats, noisy data, and artifacts were manually identified and excluded from the HRV analysis. For calculation of HRV parameters, the HRV Analysis Software, version 9.3.0 from Nevrokard (www.nevrokard.eu) was applied.

To account for long-term and for short-term HRV variations, the total monitoring period of 24 h was analyzed for time-domain parameter calculation, and selected 5-min segments were analyzed for frequency-domain parameter calculation to account for different states of activation. According to the recommendations of the Task Force of the European Society of Cardiology and North American Society of Pacing and Electrophysiology (17) and the literature for neonatal HRV measurements (18–21), the following parameters were analyzed.

Time-domain parameters [calculated from the total monitoring period (24 h)]. 1) Parameters as estimate of overall HRV: the SD of all valid NN intervals (SDNN), 2) parameters mostly influenced by parasympathetic activity: The ratio of the number of all pairs of adjacent NN intervals differing by >50 ms and the total number of RR intervals (%) (s-NN50), >27 ms (s-NN27) and >20 ms (s-NN20), the average of the hourly square root of the mean of the sum of the squares of differences between adjacent NN intervals (RMSSD), 3) parameters mostly influenced by sympathetic activity: The SD of the average of valid NN intervals (SDANN) in 5-min segments in the recording, the average of the hourly means of SDs of all NN intervals (SDNNi) in 5-min segments. As geometric index, the HRV triangular index, defined by the total number of all NN intervals divided by the height of the histogram of all NN intervals measured on a discrete scale with bins of 7.8125 ms as estimate of overall HRV was calculated.

Frequency-domain parameters (5 min segments). Power spectral analysis was calculated by fast Fourier transformation (Hamming window) in three main spectral components. To account for the neonatal physiology, frequency bandwidths were adjusted according to the literature (18,19): high frequency (HF): 0.24 to 1.04 Hz, representing parasympathetic activity; low frequency (LF): 0.04 to 0.24 Hz, representing both, sympathetic and parasympathetic activity; and very low frequency (VLF): 0.003 to 0.04 Hz with a less-defined physiologic role. Total power (ms^2) and the ratio of LF/HF considered as a marker of sympathetic-parasympathetic system balance were analyzed. Absolute values (ms^2) and normalized units (n.u.) that represent the relative value of each power component in proportion to the total power minus the VLF component were calculated. The presentation of LF and HF in n.u. emphasized the controlled and balanced behavior of the two branches of the autonomic system (17).

α-amylase measurements. Salivary α-amylase levels were analyzed from a cohort of 23 infants with antenatal betamethasone treatment and from a cohort of 40 controls that have been published previously (22). A summary of infant and maternal characteristics is shown in Table 1.

Samples were collected using a routinely performed blood sampling (heel prick test) 72 to 96 h postpartum as pain-induced stress factor. This procedure has been shown to be a significant stressor for the newborn (23,24), and salivary α-amylase has been suggested as a measure of endogenous adrenergic activity and changes in the autonomic nervous system in general (15,25) and specifically for cardiac autonomic balance (14,15,26). Saliva samples were collected from each infant 10 min before and 5 and 20 min after stress induction. Collection time was based on experiments revealing peak α-amylase responses between 5 and 10 min poststress induction (15,27). A cotton swab was placed in the neonates' mouth for a collection time of 5 min. Samples were placed in saliva collection tubes (Salivette; Sarstedt, Nümbrecht, Germany) and stored frozen at −20°C until further analysis.

The amylase 4,6-ethylidene-*p*-nitrophenyl-α,D-maltoheptaoside (EPS) method from Roche Diagnostics, Mannheim, Germany, was applied for the measurement of the α-amylase concentration in saliva. The diluted saliva samples (1 + 9) were analyzed on the Integra system 800. The assays showed good performance characteristics [intraassay coefficient of variation (CVs) < 1.0% and interassay CVs ≤ 1.3% at concentrations of 79.9 and 198 U/L].

Statistical analysis. All statistical analyses were performed with STATA 10 Statistics/Data Analysis Software (Stata Corporation, College Station, TX). Baseline characteristics of treatment and control infants were compared using the Mann-Whitney test and χ^2 test when appropriate. Because HRV parameters were not normally distributed as analyzed by the Shapiro-Francia W' test, we compared groups using the Mann-Whitney test. α-Amylase data were log transformed. The Mann-Whitney test was used for comparison of baseline α-amylase levels. Alterations of log-transformed data between baseline and poststimulation levels were analyzed by the Wilcoxon signed-rank test. To account for within-subject variation, a two-way ANOVA for repeated measurements was conducted. A stepwise multiple regression was applied to analyze the impact of gestational age at steroid administration, birth weight percentile, and gestational age at delivery as putative influencing factors on α-amylase values. The level of statistical significance of all analyses was set at $p < 0.05$.

RESULTS

Infant HRV. Betamethasone treatment was conducted at a median gestational age of 219 (178–238) d (corresponding to 31⅐ wk of gestation), and the median interval between treatment and delivery was 51 (25 to 108) d. Median birth weight and weight percentile were comparable between treatment and control groups (3040 g and 50th percentile *versus* 3260 g and 54.8th percentile). Infants of the treatment group were delivered ~2 wk earlier [260 d (37⅐ wk of gestation) *versus* 275 d (39 2/7 wk of gestation], just not reaching statistical significance ($p = 0.0504$). Infant head circumference and 5-min Apgar levels were similar ($p = 0.740$ and $p = 0.757$, respectively). A summary of the characteristics of the study population is depicted in Table 1.

For the long-term HRV analysis, the time-domain parameters SDNN and the triangular index representing estimates for

Table 2. *Long-term time-domain HRV parameters in control and treated (betamethasone) group*

	Median		Minimum		Maximum		
	Control	Treated	Control	Treated	Control	Treated	*p*
HR (bpm)	126	132	112	112	152	137	0.4218
Mean NN (ms)	478.2	455.9	395.5	438.5	538.2	534.4	0.3976
Triangular index	20	17	8	10	34	28	0.1792
s-NN50 (%)	2.46	2.13	.14	.26	11.56	12.76	0.8519
s-NN27 (%)	10.97	10.43	1.71	2.54	29.92	29.05	0.6844
s-NN20 (%)	20.6	18.6	4.61	7.09	42.4	38.5	0.8176
SDNN (ms)	69.92	65.82	31.88	39.83	108.4	96.97	0.2579
SDNNI (ms)	42.58	42.62	18.79	27.1	61.38	69.55	0.7836
r-MSSD (ms)	19.4	18.04	10.54	11.74	36.27	41.31	1.0000
SDANN (ms)	52.0	51.25	24.11	21.96	85.18	70.96	0.1838

Table 3. *Short-term frequency-domain HRV parameters in control and treated (betamethasone) group*

	Active state			Resting state		
	Control	Treated	*P*	Control	Treated	*p*
HF (ms^2)	318.75 (15.80–2251.78)	252.12 (12.77–911.21)	0.3242	51.92 (14.69–184.32)	58.31 (9.20–166.71)	0.7853
HF (n.u.)	0.48 (0.20–0.99)	0.42 (0.19–2.26)	0.3478	0.18 (0.08–0.31)	0.17 (0.07–0.30)	0.8204
LF (ms^2)	263.61 (14.48–2246.17)	402.24 (42.73–1185.95)	0.7424	263.61 (14.48–2246.17)	402.24 (42.73–1185.95)	0.8916
LF (n.u.)	0.52 (0.01–0.80)	0.61 (0.24–2.03)	0.1522	0.82 (0.69–0.92)	0.83 (0.70–0.93)	0.8204
VL (ms^2)	79.96 (8.63–1482.97)	147.68 (8.87–170.47)	0.3981	317.36 (47.01–899.14)	214.62 (55.83–1030.80)	0.0565
Total power (ms^2)	857.90 (38.91–3612.32)	818.24 (75.00–2967.94)	0.4668	791.37 (157.43–1495.71)	618.09 (150.45–1623.06)	0.2288
LF/HF	1.06 (0.32–4.30)	1.38 (0.32–4.30)	0.2699	4.47 (2.24–11.68)	4.82 (2.34–13.08)	0.8204

Values are given as medians (range).

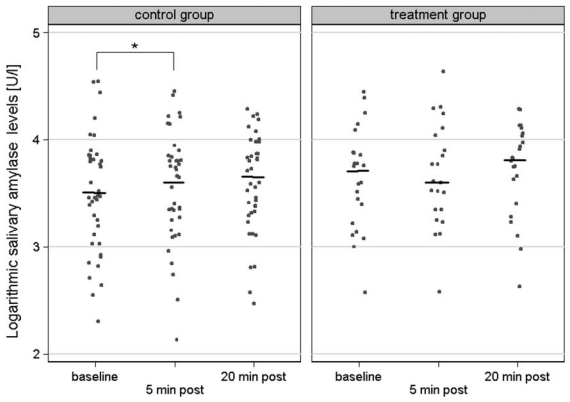

Figure 1. Individual log-transformed salivary levels and medians for α-amylase in control ($n = 40$) and study group ($n = 23$) newborns before (baseline) and after (5 and 20 min after) application of the stress stimulus. (left = control; right = study group). Horizontal line indicates median levels. *$p < 0.05$.

overall HRV were not significantly different in the treatment group compared with the controls ($p = 0.258$ and $p = 0.179$, respectively). Similarly, parameters, mostly influenced by sympathetic activity, such as SDANN and SDNNi were similar ($p = 0.184$ and $p = 0.784$). No significant differences were also found for estimates of vagal tone as RMSSD ($p = 1.0$) and for the proportion of pairs of adjacent NN intervals differing by >50, >27, and >20 ms ($p = 0.852$, $p = 0.684$, and $p = 0.818$; Table 2). Short-term HRV analysis during active and resting states calculating frequency-domain parameters confirmed these results. Although there was a significant difference between active and resting states within each group as expected (data not shown), comparison of parameters between similar states of activation were not significantly different when calculating absolute and normalized values. Accordingly, HF, representing vagal activity; LF, representing both sympathetic and vagal activity; VLF, representing total power; and LF/HF, representing sympathovagal balance, all were not significantly different from control infants (Table 3). The likelihood to miss a putative significant difference by our sample size is very low [5.2% ($\alpha = 0.8$, $\beta = 0.05$) for RMSSD and LF/HF (n.u.) resting state].

Infant α-amylase levels. Betamethasone treatment was conducted at a median gestational age of 201 (173–235) d, and the median interval to delivery was 55 (16–102) d. Median birth weight and weight percentile were comparable in both groups ($p = 0.269$ and $p = 0.617$, respectively). Gestational age at delivery was 266 (242–284) d in the treatment group and 273 (240–294) d in the control group ($p = 0.201$). Head circumference and 5-min Apgar scores were similar ($p = 0.583$ and $p = 0.517$, respectively). A summary of the characteristics of the study population is shown in Table 1.

Median baseline levels for α-amylase were slightly higher but not significantly different in the treatment group when compared with controls ($p = 0.3040$, Fig. 1). α-Amylase levels in control infants increased marginally, just reaching the level of statistical significance ($p = 0.045$) at the time point 5-min poststimulation, whereas in infants with betamethasone treatment, no significant alteration of α-amylase release was observed ($p = 0.408$). A two-way ANOVA confirmed that there was a significant alteration of α-amylase in the control ($p = 0.0266$) but not in the study group ($p = 0.183$) after the stress stimulus. No significant influence of gestational age at steroid administration ($p = 0.82$), newborn weight ($p = 0.079$), and gestational age at delivery ($p = 0.46$) was observed applying a multiple stepwise regression model.

DISCUSSION

We have shown that cardiac autonomic system balance is preserved in the neonate after a single course of antenatal maternal betamethasone treatment for fetal lung maturation in pregnancies at risk for preterm delivery.

There is experimental evidence that intrauterine exposure to synthetic glucocorticoids affects balance and activity of the autonomic cardiovascular system. In adult male guinea pigs with repeated prenatal exposure to dexamethasone, arterial blood pressure was significantly elevated (28). Even a single course of glucocorticoid treatment produced a sustained elevation of blood pressure and altered baroreceptor heart rate response in fetal sheep and the baboon (29–32) and increased central and peripheral vascular resistance (29,30,33). However, others only found transient hypertension after maternal dexamethasone treatment in fetal sheep (34). In the nonhuman primate, multiple antenatal dexamethasone exposure resulted in elevated systolic and diastolic blood pressure at the age of 12 mo (35).

The connection of prenatal glucocorticoid treatment and increased blood pressure in the human is less clear. In the neonate (36) and in a cohort of preterm children at the age of 14 y (37), blood pressure was significantly increased, whereas other data have shown that blood pressure alterations may rather be transient and normalize within the first few weeks of life (38). Large follow-up studies did not find significant alterations in blood pressure in nonstrained conditions at the age of 20 and 30 y (10,11).

Data addressing more specifically the cardiac autonomic system reveal persistent abnormalities of cardiac noradrenergic innervation after intrauterine exposure to exogenous steroids in the rat (6) and alterations of cardiac intracellular β-adrenoceptor signaling (7). Furthermore, centrally located systems involved in cardioregulation such as the brainstem norepinephrine system seem to undergo premature maturation in response to prenatal synthetic glucocorticiod exposure (39). There is some evidence that betamethasone may even have a direct effect on the cardiac pacemaker (40,41). It has been shown that infants with antenatal betamethasone treatment display a significant increase in heart rate in response to a stressor when compared with control infants (5).

To our knowledge, a systematic analysis of HRV parameters according to standardized methods (17) so far has not been performed in individuals exposed to prenatal glucocorticoid treatment. HRV is a well-established noninvasive measure of cardiac autonomic control that has been shown to be related to hypertension (12,13) and to predict future adverse cardiovascular events in adults (42). HRV analyses have been suggested for putative prognostic use in children (43). We did not find significant alterations of any HRV parameter analyzed in neonates after antenatal betamethasone treatment. Studies measuring intrauterine FHR variation after steroid administration have produced inconsistent results. Mulder *et al.* (3), analyzing FHR short-term and long-term variation, found reductions in FHR variation after maternal betamethasone administration. In contrast, treatment with dexamethasone resulted in a significant rise in short-term FHR variation (4). However, both effects were only transiently observed.

We conducted our study in the neonatal period because our aim was to find out whether putative intrauterine steroid effects ultimately transform into permanent postnatal alterations of the cardiac autonomic system and to exclude postnatal-induced influencing factors of system adaptation. This aspect is even more important in consideration that diseases susceptible to intrauterine origin such as hypertension and type 2 diabetes will exhibit multiple system alterations at the time when they become apparent. For the development of prevention programs for arterial hypertension and type 2 diabetes, the initiating systems have to be identified and discriminated from systems secondarily affected. In this study, putative short-term effects could be excluded as the median interval between betamethasone treatment and delivery was more than 7 wk. Our findings provide new evidence that at the level of the cardiac autonomic subunit, at least, a single course of betamethasone does not primarily affect sympathetic cardiac balance.

As early effects may only become apparent during events with increased demand for adaptive regulation, we supplemented our electrophysiological studies with a neuroendocrinological approach by analyzing salivary α-amylase levels during resting conditions and after a stressful stimulus. Salivary α-amylase is secreted by acinar cells in the salivary glands that are richly innervated by both sympathetic and parasympathetic nerve fibers, influencing the release of α-amylase by classic neurotransmitters (44). Studies in humans and animals have suggested that the activation of the autonomic nervous system leads to a high activity of salivary α-amylase (25,45–47). Furthermore, α-amylase levels have been found to be associated with cardiovascular physiology and are suggested to be a surrogate for cardiovascular autonomic system balance (26). Bosch *et al.* (14) found a significant negative correlation between the parasympathetic-influenced HRV parameter RMSSD and α-amylase levels during stress induction in adults. Furthermore, a positive correlation between α-amylase levels and LF/HF as surrogate for sympathetic tone has been shown (15), thereby making this parameter a promising indicator for cardiac autonomic function. Although studies on cardiovascular autonomic physiology using α-amylase measurements have not been validated in neonates, several analyses from children support the strong relationship between salivary α-amylase and sympathetic/parasympathetic nervous system activation in younger individuals (26). Interestingly, we found stimulated α-amylase levels in betamethasone-exposed neonates rather decreased when compared with controls although statistical significance was only marginal ($p = 0.045$). One rather had expected increases in α-amylase levels as response to an increased sympathetic stress reaction if betamethasone treatment had affected the system.

The slightly attenuated α-amylase response might be explained by previous findings about the stress-regulating hypothalamo-pituitary-adrenal (HPA) axis. Indeed, we and others have shown that a single course of antenatal betamethasone administration causes sustained suppressive effects of HPA

reactivity in these infants (5,22,48). We speculate a nonsignificant increase in α-amylase levels may be the result of inadequate HPA system sensitivity rather than direct effects of steroid exposure on the sympathetic system because these systems are closely interconnected. A stepwise multiple regression was conducted to exclude putative influencing factors such as gestational age at steroid administration, newborn weight, and gestational age at delivery, not revealing a significant influence on α-amylase results.

We analyzed a relatively homogenous collective excluding isolated effects of severe prematurity, pregnancy complications such as intrauterine growth restriction or preeclampsia in addition to maternal substance abuse and diseases. Nevertheless, although not statistically significant, there was a difference of 15 d in gestational age between the control and the treatment group. This may, even though near or at term, entail a difference in perinatal maturation with a putative influence on the cardiac autonomic system. It has been shown that with increasing maturation of the sympathetic nervous system during the course of pregnancy, there is a shift of the sympathetic/parasympathetic balance toward parasympathetic tone (49–51). The time-domain parameter RMSSD has been shown to demonstrate highest correlation to gestational age (51), and for frequency-domain parameters, gestational age-dependent alterations of LF and HF parameters have only been found to be significant during resting and not during active states (49). Therefore, we analyzed our data for a putative influence of gestational age on these parameters and conducted a regression analysis. We found adjusted R-squared values for RMSSD of 0.10, for LF/HF (resting state) of 0.00, and for LF/HF (active) of 0.02, indicating no relevant effect of the difference in gestational age in our groups. Thus, our data strongly support the notion that at least the cardiac autonomic subunit of the sympathetic system is not permanently affected *in utero* by antenatal steroid treatment for lung maturation.

It is important to note that the sympathetic nervous system is composed of multiple function-specific subunits (52) and the programming of sympathetic nervous system function is believed to occur regionally rather than on a global basis, suggesting that subdivisions of the sympathetic nervous system may be influenced by different sets of environmental variables (53), thus aspects such as vascular bed regulation may be affected differently.

We cannot exclude that our methods were not sensitive enough to expose small alterations in cardiac sympathetic system balance that may only become apparent with increasing system maturation during infant and adolescent development. Even though long-term analyses of antenatal steroid treatment in young adults did not find sympathoadrenergic connected pathologies (10), these diseases may not be present until later ages at the time they usually become apparent. In fact, putative early markers of sympathetic cardiac activation such as HRV parameters have not been applied in these studies.

In conclusion, despite evidence from animal models, it seems that the neonatal cardiac autonomic system is primarily unaffected by a single course of betamethasone treatment for lung maturation. However, the impact of multiple doses is not known.

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Discussion and future perspectives

Our research has shown that the development of the HPA axis appears to be vulnerable towards antenatal challenge whereas the cardiac SNS may be developmentally more stable.
In the context of our work, several considerations are to be addressed and current knowledge on these topics will be presented.

1. Do antenatal alterations, once established persist during life?
2. Are there specific time windows for the vulnerability of the systems?
3. Is there a gender difference?
4. What happens at the other end of the U-shaped birth weight risk curve?
5. Are there putative preventive / therapeutic options?

Do antenatal alterations, once established persist during life?

Our studies have shown that both intrauterine growth restriction and iatrogenic corticosteroid application during intrauterine development result in a blunted reactivity of the HPA axis in the newborn (Schaffer, Luzi et al. 2009; Schaffer, Muller-Vizentini et al. 2009). One may rather have expected an overreaction of this system in consideration of the activated state under unfavorable intrauterine conditions (Economides, Nicolaides et al. 1988; Goland, Jozak et al. 1993). Furthermore, a variety of studies in adults with low birth weight have shown signs of HPA axis hyperactivity either by measuring non-stimulated or stimulated cortisol levels as lately summarized by Kajantie (Kajantie 2006). Thus it appears that the HPA axis is subject to functional plasticity during the interval between the initiating event and the endpoint. Few studies have analyzed HPA axis development during the first years of human life. However, it appears that healthy children dampen their cortisol responses to stress during

the first year of life (Gunnar, Brodersen et al. 1996; Larson, White et al. 1998) representing a physiologic stress hyposensitive period during development. The finding in our studies of a blunted reactivity of the HPA axis towards a stress event may point to an acceleration of HPA axis system maturation, putatively cortisol induced (Fowden 1995), that may in consequence represent an inadequate developmental time frame for steps of proper HPA axis establishment. It has been speculated that stress hyporesponsive periods may be connected with the circadian day-night periodicity but this notion could not be confirmed in a later study (Gunnar, Brodersen et al. 1996; Larson, White et al. 1998). Data from animal models suggest that all levels of the HPA axis may be involved as alterations in adrenal capacity and responsiveness to ACTH and alterations in various hypothalamic receptor expression levels have been found that may be responsible for a hyporesponsive HPA axis (Rosenfeld, Suchecki et al. 1992; Challis, Sloboda et al. 2001; Gardner, Jamall et al. 2004). As such, blunted HPA axis responses have been found in sheep fetuses during late gestation after early intrauterine stress. The authors demonstrated in these animals a decreased expression of CRH in the hypothalamus. Additionally, the GR receptor in the pituitary and in the adrenal ACTH receptor were decreased, thereby impairing HPA sensitivity as well as feedback regulation (Challis, Sloboda et al. 2001) Selective analyses of different levels within the feed-back control system in the human will provide further insight in HPA axis-dependent blunted stress response situations and its conversion into hyperactivity. It is not clear at what time point the switch of a hypo-responsive HPA axis towards a hyper-responsive state takes place and which levels of the axis are involved. It has been speculated that increased ACTH levels during a period of decreased adrenal capacity may prime the adrenal gland to increase its responsiveness towards future stressors (Rosenfeld, Suchecki et al. 1992). Other factors, such as prostaglandins have been suggested to be involved in adrenal glucocorticoid liberation in this context (McMillen, Adams et al. 2001). In summary, intrauterine induced alterations in the regulation of the HPA axis leads to a state of early hypo-responsiveness in the newborn

which appears to be transformed intro a hyperactivity during postnatal life. The exact mechanisms involved are to be further analyzed.

Are there specific time windows for the vulnerability of the systems?

It is obvious that vulnerability of systems may depend upon certain sensitive periods in their development. This has most impressively been shown in sheep with a pregnancy duration of approximately 150 days, where short term dexamethasone exposure for 48h during early pregnancy (day 27) did result in hypertension during adulthood while those animals exposed at 64 days remained normotensive and glucose tolerance was preserved in all animals (Gatford, Wintour et al. 2000). In contrast, single or repeated doses of betamethasone after 100 days of gestation induced an altered glucose metabolism and normotensive sheep at 1 year of age (Moss, Sloboda et al. 2001). Likewise, alterations of the HPA axis responsiveness have been observed after glucocorticoid exposure during late gestation (day 104-125) but not when exposure happened during early gestation (day 25-45) (Moritz, Butkus et al. 2002; Sloboda, Moss et al. 2007). Vulnerable periods of systems in the human are less well defined. One may speculate that early- vs. late intrauterine fetal growth restriction as well as early (24 weeks of gestation) vs. late (33 weeks of gestation) betamethasone exposure induce different patterns of risk profiles. Lately, this question has been addressed in an evaluation of the historical cohort of the Dutch Winter famine. Interestingly, cardiac disease, body mass index and disturbed glucose was especially increased when exposure towards famine had been occurred during early gestation as to compared to mid- or late gestation (Ravelli, van Der Meulen et al. 1999; de Rooij, Painter et al. 2006; Painter, de Rooij et al. 2006). Thus, it appears that unfavorable conditions during early pregnancy may be even more profound. Indeed, the periconceptional period has been shown to already impact vascular function in adult sheep (Torrens, Snelling et al. 2009).

Thus, each phase of intrauterine development is likely to carry different vulnerabilities and unfavorable intrauterine conditions may have the potential for variable long-term consequences for different organ systems.

Is there a gender difference?

There is evidence from human and animal studies, that gender may be relevant for the development of diseases of intrauterine origin. In the rat, a gender specific lowering of insulin sensitivity and hyperinsulinaemia has been shown in adult males but not females after maternal protein restriction during pregnancy and lactation (Sugden and Holness 2002). Others have shown that antenatal glucocorticoid exposure resulted in gender specific cardiovascular and metabolic physiology alterations as male rats exhibited increased ACTH, corticosterone, postprandial insulin-glucose ratios and hepatic gluconeogenic enzyme expression while female rats were hypertensive displaying a renin-angiotensin system activation (O'Regan, Kenyon et al. 2004). In the adult pig, low birth weight is associated with increased cortisol responses to hypoglycaemia in both genders but involves specific changes in the female adrenal and male hypothalamic-pituitary region (Poore and Fowden 2003). Differential vulnerability of glucocorticoid receptor expression in the hippocampus, hypothalamus and pituitary has been suggested (Matthews 2000; Seckl 2004) and it appears that females may be more sensitive towards HPA axis programming than males (Weinstock, Matlina et al. 1992; McCormick, Smythe et al. 1995). On the other hand, juvenile males have been shown to be more sensitive towards antenatal glucocorticoids than females at the level of the pituitary and adrenal in the guinea pig (Owen and Matthews 2007). Associations between LBW and increased HPA axis activation have not been found to be different in adult men and women albeit the female HPA axis appears to be generally more responsive than the male (Reynolds, Walker et al. 2005). However, in a cohort of 7-9 year old children, boys born with

a low birth weight exhibited raised arterial pressure and systemic vascular resistance, particularly after strain while this association was not found in girls. The girls instead exhibited increased cardiac sympathetic nervous system activation when born LBW. At the same time, cortisol responses to a psychiological stress test was inversely correlated with birth weight in boys but not in girls (Jones, Beda et al. 2005; Jones, Godfrey et al. 2005). In a cohort of Australian low birth weight adults, signs for cardiovascular autonomic control were altered in women but not in men (Jones, Beda et al. 2007). Gender differences in the association between birth weight and total cholesterol have been found (Lawlor, Owen et al. 2006). We did not find gender differences in our studies in neonates, however, it seems that gender-specific cortisol reactivity in general depends on different levels of corticosteroid-binding globulin and sex steroids and therefore gender-specific hormonal influences may manifest later during adolescence and adulthood (Kudielka, Buske-Kirschbaum et al. 2004). In fact, environmental or nutritional factors have been suggested to lead to gender specific epigenetic modulation of gene expression profiles through gender-specific methylation of CpC regions and histone modifications (Gabory, Attig et al. 2009).

What happens at the other end of the U-shaped birth weight risk curve?

A variety of studies have reported an U-shaped relationship between birth weight and the risk for metabolic disorders. Excessive energy supply and hyperinsulinaemia may equally constitute an unfavorable intrauterine environment. Accordingly, large for gestational age in combination with maternal gestational diabetes has been shown to demonstrate a significant risk factor for the development of the metabolic syndrome in adolescence (Boney, Verma et al. 2005). Similar results have been found in a Chinese population (Wang, Liang et al. 2007). A meta-analysis including 14 studies confirmed a U-shaped relation between birth weight and subsequent risk of type 2 diabetes (Harder, Rodekamp et al. 2007). Furthermore, increased

systolic and mean arterial pressure has been found in adolescent offspring of diabetic pregnancies (Silverman, Rizzo et al. 1991; Bunt, Tataranni et al. 2005).

The mechanisms underlying these findings are under investigation and experimental evidence suggest that insulin and leptin exert dominant influences on central energy-regulating neural networks involving hypothalamic regions (Muhlhausler, Adam et al. 2006). Indeed, early overfeeding in rats resulted in a resistance towards insulin and leptin of central hypothalamic regions involved in regulation of feeding behaviour and thus body weight (Davidowa and Plagemann 2000; Davidowa and Plagemann 2007). Interestingly, it has recently been shown in the rat that early overfeeding induced a functional alteration in DNA methylation of the hypothalamic gene promoter of the anorexigenic neurohormone proopiomelanocortin that is involved in the regulation of food intake and body weight by insulin and leptin mediation (Plagemann, Harder et al. 2009).

There is strong evidence, that renal and vascular mechanisms are additionally involved. As such, intrauterine exposure to hyperglycemia did result in a significant reduction in the number of nephrons in the rat offspring (Amri, Freund et al. 1999). Furthermore, macrosomic newborns of diabetic mothers displayed an increased aortic intima-media thickness as compared to controls (Akcakus, Koklu et al. 2007) and there is evidence for precocious artherosclerosis (Skilton 2008).

Hyperglycemia has been shown to significantly decrease angiogenesis in the chicken embryo putatively involving apoptosis and impaired response to pro-angiogenic factors (Larger, Marre et al. 2004; Di Marco, Alam et al. 2008) and adult male rats of diabetic pregnancies exhibited a permanent decrease of vascular nitric oxide (NO) dependent vasodilatation (Rocha, Gomes et al. 2005).

Lately, it has been shown in the mouse model that diet induced obesity had persistent effects on cardiovascular and metabolic function in the offspring such as hypertension and an abnormally high heart rate with signs indicative for endothelial dysfunction and diminished baroreceptor sensitivity (Samuelsson, Matthews et al. 2008). Whether the cardiac sympathetic nervous system is involved in these effects is currently under investigation.

Are there options for reversibility / therapeutic strategies?

As regulating systems such as the HPA axis contain a certain degree of plasticity during postnatal development (Gunnar, Brodersen et al. 1996; Larson, White et al. 1998), the question arises as to whether intrauterine induced unfavorable phenotypic effects may be reversed during the postnatal development. Indeed, the HPA axis appears to be a biological substrate for the interaction of prenatal and postnatal events. As such, repeated maternal restraint during the last week of gestation in rats produced a prolonged stress-induced cortisol response in the adult offspring. Interestingly, when offspring were raised by a foster mother, adoption at birth completely reversed the effects of prenatal stress. These findings were associated with rescued mineralocorticoid receptors in the hypothalamus probably restoring negative feedback mechanisms (Maccari, Piazza et al. 1995). Adoption has been shown to enhance maternal behavior towards the pub as compared to the biological mother.

In fact, epigenetic alteration of a hippocampal glucocorticoid receptor gene promoter has been shown to occur as a result of increased licking and grooming during the neonatal period of the rat undermining the theory of behavioral programming during this time through glucocorticoid receptor expression (Weaver, Cervoni et al. 2004).

However, one has to consider that in species such as the rodent important steps of neuroendocrine development occurs in the postnatal period while in primates, sheep and guinea pigs major brain growth and neuroendocrine maturation happens in utero (Dobbing

and Sands 1979; Matthews 1998; Challis, Matthews et al. 2000; Dent, Smith et al. 2000). Accordingly, manipulations may occur during different stages of neuroendocrine development depending on the species that is being analyzed.

Nevertheless, even in the mature adult brain of the rat, epigenetic programming and thus phenotype could be shown to be reversible by treatment with Trichostatin A a Histone deacetylase inhibitor triggering active genomic demethylation (Ou, Torrisani et al. 2007). As such, Trichostatin A administration was able to completely reverse maternal induced hyperreactivity of the HPA axis in the adult offspring and this was accompanied by a significant increase in hippocampal glucocorticoid receptor levels pointing to a restored negative feedback mechanism (Weaver, Cervoni et al. 2004). On the other hand, methionine treatment resulting in increased methylation of the glucocorticoid receptor promoter produced the pathologic phenotype in previously healthy adult rats (Weaver, Champagne et al. 2005). Thus it has been suggested that dietary changes in methyl contents may effect epigenetic programming (McGowan, Meaney et al. 2008).

Several studies have provided evidence for a putative beneficial effect of leptin-level manipulations. This adipokin, as described above, is involved in hypothalamic regulation, food intake and energy expenditure and is believed to be significantly involved in the perinatal programming of the metabolic syndrome. As such, neonatal leptin treatment prevented development of the metabolic syndrome in a model of maternal undernutrition in the rat offspring (Vickers, Gluckman et al. 2005). Likewise, leptin supplementation during the first 10 days of life partially reversed the IUGR phenotype of the low birth weight piglet (Attig, Djiane et al. 2008). Adversely, supplementation of ώ3 fatty acids during postnatal nutrition prevented hyperleptinaemia and hypertension in the adult offspring of dexamethasone treated rats (Wyrwoll, Mark et al. 2006). Apparently, leptin effects are complex and they have been shown to be especially sensitive to pre- and postnatal nutritional

status (Vickers, Gluckman et al. 2008). Clearly, further studies are required to ascertain the exact role of leptin in these settings.

Accumulating knowledge on possibilities to restore a misrouted organism towards normal balance gives rise to the hope that early interventions, including behavioral and nutritional strategies, may ultimately be able to prevent, reverse or at least alleviate the burden of the risk to develop diseases of intrauterine origin in later life.

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